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# (54) Title: PLANT DESATURASES - COMPOSITIONS AND USES

#### (57) Abstract

By this invention, compositions and methods of use of plant desaturase enzymes, especially  $\Delta$ -9 desaturases, are provided. Of special interest are methods and compositions of amino acids and nucleic acid sequences related to biologically active plant desaturases as well as sequences, especially nucleic acid sequences, which are to be used as probes, vectors for transformation or cloning intermediates. Biologically active sequences may be found in a sense or anti-sense orientation as to transcriptional regulatory regions found in various constructs.

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## PLANT DESATURASES -COMPOSITIONS AND USES

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This application is a continuation-in-part of USSN 07/494,106 filed on March 16, 1990 and a continuation-in-part of USSN 07/567,373 filed on August 13, 1990 and a continuation-in-part of USSN 07/615,784 filed on November 14, 1990.

#### Technical Field

The present invention is directed to desaturase enzymes relevant to fatty acid synthesis in plants,

15 enzymes, amino acid and nucleic acid sequences and methods related thereto, and novel plant entities and/or oils and methods related thereto.

#### INTRODUCTION

## 20 Background

Novel vegetable oils compositions and/or improved means to obtain or manipulate fatty acid compositions, from biosynthetic or natural plant sources, are needed. Depending upon the intended oil use, various different oil compositions are desired. For example, edible oil sources containing the minimum possible amounts of saturated fatty acids are desired for dietary reasons and alternatives to current sources of highly saturated oil products, such as tropical oils, are also needed.

One means postulated to obtain such oils and/or modified fatty acid compositions is through the genetic engineering of plants. However, in order to genetically engineer plants one must have in place the means to transfer genetic material to the plant in a stable and heritable manner. Additionally, one must have nucleic acid sequences capable of producing the desired phenotypic result, regulatory regions capable of directing the correct application of such sequences, and the like. Moreover, it

should be appreciated that to produce a desired modified oils phenotype requires that the Fatty Acid Synthetase (FAS) pathway of the plant is modified to the extent that the ratios of reactants are modulated or changed.

Higher plants appear to synthesize fatty acids via a common metabolic pathway in plant plastid organelles (i.e., chloroplasts, proplastids, or other related organelles) as part of the FAS complex. Outside of plastid organelles, fatty acids are incorporated into triglycerides and used in plant membranes and in neutral lipids. In developing seeds, where oils are produced and stored as sources of energy for future use, FAS occurs in proplastids.

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The production of fatty acids begins in the plastid with the reaction between Acyl Carrier Protein (ACP) and acetyl-CoA to produce acetyl-ACP. Through a sequence of cylical reactions, the acetyl-ACP is elongated to 16- and 18- carbon fatty acids. The longest chain fatty acids produced by the FAS are 18 carbons long. Monunsaturated fatty acids are also produced in the plastid through the action of a desaturase enzyme.

Common plant fatty acids, such as oleic, linoleic and  $\alpha$ -linolenic acids, are the result of sequential desaturation of stearate. The first desaturation step is the desaturation of stearoyl-ACP (C18:0) to form oleoyl-ACP (C18:1) in a reaction often catalyzed by a  $\Delta$ -9 desaturase, also often referred to as a "stearoyl-ACP desaturase" because of its high activity toward stearate the 18 carbon acyl-ACP. The desaturase enzyme functions to add a double bond at the ninth carbon in accordance with the following reaction (I):

Stearoyl-ACP + ferredoxin(II) +  $O_2$  +  $2H^+$  -> oleoyl-ACP + ferredoxin(III) +  $2H_2O$ .

Δ-9 desaturases have been studied in partially purified preparations from numerous plant species. Reports indicate that the protein is a dimer, perhaps a homodimer, displaying a molecular weight of 68 kD (±8 kD) by gelfiltration and a molecular weight of 36 kD by SDS-polyacrylamide gel electrophoresis.

In subsequent sequential steps for triglyceride production, polyunsaturated fatty acids may be produced. These desaturations occur outside of the plastid as a result of the action of membrane-bound enzymes. Additional double bonds are added at the twelve position carbon and thereafter, if added, at the 15 position carbon through the action of  $\Delta$ -12 desaturase and  $\Delta$ -15 desaturase, respectively.

Obtaining nucleic acid sequences capable of producing 10 a phenotypic result in FAS, desaturation and/or incorporation of fatty acids into a glycerol backbone to produce an oil is subject to various obstacles including but not limited to the identification of metabolic factors of interest, choice and characterization of a protein source with useful kinetic properties, purification of the 15 protein of interest to a level which will allow for its amino acid sequencing, utilizing amino acid sequence data to obtain a nucleic acid sequence capable of use as a probe to retrieve the desired DNA sequence, and the preparation of constructs, transformation and analysis of the resulting 20 plants.

Thus, the identification of enzyme targets and useful plant sources for nucleic acid sequences of such enzyme targets capable of modifying fatty acid compositions are Ideally, an enzyme target will be amenable to one 25 needed. or more applications alone or in combination with other nucleic acid sequences relating to increased/decreased oil production, the ratio of saturated to unsaturated fatty acids in the fatty acid pool, and/or to novel oils compositions as a result of the modifications to the fatty 30 acid pool. Once enzyme target(s) are identified and qualified, quantities of protein and purification protocols are needed for sequencing. Ultimately, useful nucleic acid constructs having the necessary elements to provide a phenotypic modification and plants containing such 35 constructs are needed.

#### Relevant Literature

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A 200-fold purification of Carthamus tinctorius ("safflower") stearoyl-ACP desaturase was reported by McKeon & Stumpf in 1982, following the first publication of their protocol in 1981. McKeon, T. & Stumpf, P. J.Biol.Chem. (1982) 257:12141-12147; McKeon, T. & Stumpf, P. Methods in Enzymol. (1981) 71:275-281.

## BRIEF DESCRIPTION OF THE DRAWINGS

10 Fig. 1 provides amino acid sequence of fragments relating to C. tinctorius desaturase. Fragments F1 through F11 are also provided in the sequence listing as SEQ ID NO: 1 through SEQ ID NO: 11, respectively. Each fragment represents a synthesis of sequence information from peptides originating from different digests which have been 15 matched and aligned. In positions where there are two amino acids indicated, the top one corresponds to that found in the translation of the cDNA; the lower one was detected either as a second signal at the same position of 20 one of the sequenced peptides, or as a single unambiguous signal found in one or more of the overlapping peptides comprising the fragment. Residues in F9 shown in lower case letters represent positions where the called sequence does not agree with that predicted from the cDNA, but where 25 the amino acid assignment is tentative because of the presence of a contaminating peptide. The standard one letter code for amino acid residues has been used. X represents a position where no signal was detectable, and which could be a modified residue. F1 corresponds to the N-terminal sequence of the mature protein. 30 The underlined region in F2 is the sequence used in designing PCR primers for probe synthesis.

Fig. 2 provides a cDNA sequence (SEQ ID NO: 12) and the corresponding translational peptide sequence (SEQ ID NO: 13) derived from *C. tinctorius* desaturase. The cDNA sequence includes both the plastid transit peptide encoding sequence and the sequence encoding the mature protein.

Fig. 3 provides cDNA sequence of Ricinus communis desaturase. Fig. 3A provides preliminary partial cDNA sequence of a 1.7 kb clone of R. communis desaturase (SEQ ID NO: 14). The sequence is from the 5' end of the clone. Fig. 3B provides the complete cDNA sequence of the approximately 1.7 kb clone (SEQ ID NO: 15) and the corresponding translational peptide sequence (SEQ ID NO: 16).

Fig. 4 provides sequence of Brassica campestris

desaturase. Fig. 4A represents partial DNA sequence of a

1.6 kb clone pCGN3235 (SEQ ID NO: 17), from the 5' end of
the clone. Fig. 4B represents partial DNA sequence of a

1.2 kb clone, pCGN3236, from the 5' end of the clone (SEQ
ID NO: 18). Initial sequence for the 3' ends of the two B.

campestris desaturase clones indicates that pCGN3236 is a
shorter cDNA for the same clone as pCGN3235. Fig. 4C
provides complete cDNA sequence of B. campestris desaturase
above, pCGN3235 (SEQ ID NO: 19) and the corresponding
translational peptide sequence (SEQ ID NO: 20).

Fig. 5 provides preliminary partial cDNA sequence of Simmondsia chinensis desaturase (SEQ ID NO: 43). The translated amino acid sequence is also shown.

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Fig. 6 shows the design of forward and reverse primers (SEQ ID NO: 21 through SEQ ID NO: 26) used in polymerase chain reaction (PCR) from the sequence of *C. tinctorius* desaturase peptide "Fragment F2" (SEQ ID NO: 2).

Fig. 7 provides maps of desaturase cDNA clones showing selected restriction enzyme sites. Fig. 7A represents a *C. tinctorius* clone, Fig. 7B represents a *R. communis* clone, and Fig. 7C represents a *B. campestris* clone.

Fig. 8 provides approximately 3.4 kb of genomic sequence of Bce4 (SEQ ID NO: 27).

Fig. 9 provides approximately 4 kb of genomic sequence of Bcg 4-4 ACP sequence (SEQ ID NO: 28).

Fig. 10 provides a restriction map of cloned  $\lambda$ CGN 1-2 showing the entire napin coding region sequence as well as extensive 5' upstream and 3' downstream sequences (SEQ ID NO: 29).

### SUMMARY OF THE INVENTION

By this invention, compositions and methods of use of plant desaturase enzymes, especially  $\Delta$ -9 desaturases, are provided. Of special interest are methods and compositions of amino acids and nucleic acid sequences related to biologically active plant desaturases as well as sequences, especially nucleic acid sequences, which are to be used as probes, vectors for transformation or cloning intermediates. Biologically active sequences may be found in a sense or anti-sense orientation as to transcriptional regulatory regions found in various constructs.

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A first aspect of this invention relates to C. tinctorius  $\Delta$ -9 desaturase substantially free of seed storage protein. Amino acid sequence of this desaturase is provided in Fig. 2 and as SEQ ID NO: 13.

DNA sequence of *C. tinctorius* desaturase gene (SEQ ID NO: 12) is provided, as well as DNA sequences of desaturase genes from a *Ricinus* (SEQ ID NO: 14 and SEQ ID NO: 15) a *Brassica* (SEQ ID NO: 17 through SEQ ID NO: 19) and a *Simmondsia* (SEQ ID NO: 43) plant.

In yet a different embodiment of this invention, plant desaturase cDNA of at least 10 nucleotides or preferably at least 20 nucleotides and more preferably still at least 50 nucleotides, known or homologously related to known  $\Delta$ -9 desaturase(s) is also provided. The cDNA encoding precursor desaturase or, alternatively, biologically active, mature desaturase is provided herein.

Methods to use nucleic acid sequences to obtain other plant desaturases are also provided. Thus, a plant desaturase may be obtained by the steps of contacting a nucleic acid sequence probe comprising nucleotides of a known desaturase sequence and recovery of DNA sequences encoding plant desaturase having hybridized with the probe.

This invention also relates to methods for obtaining plant  $\Delta$ -9 desaturase by contacting an antibody specific to a known desaturase, such as C. tinctorius stearoyl-ACP

desaturase, with a candidate plant stearoyl-ACP desaturase under conditions conducive to the formation of an antigen:antibody immunocomplex and the recovery of the candidate plant stearoyl-ACP desaturase which reacts thereto.

In a further aspect of this invention DNA constructs comprising a first DNA sequence encoding a plant desaturase and a second DNA sequence which is not naturally found joined to said plant desaturase are provided. This invention also relates to the presence of such constructs in host cells, especially plant host cells. In yet a different aspect, this invention relates to transgenic host cells which have an expressed desaturase therein.

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Constructs of this invention may contain, in the 5' to 15 3' direction of transcription, a transcription initiation control regulatory region capable of promoting transcription in a host cell and a DNA sequence encoding plant desaturase. Transcription initiation control regulatory regions capable of expression in prokatyotic or 20 eukaryotic host cells are provided. Most preferred are transcription initiation control regions capable of expression in plant cells, and more preferred are transcription and translation initiation regions preferentially expressed in plant cells during the period of lipid accumulation. The DNA sequence encoding plant 25 desaturase of this invention may be found in either the sense or anti-sense orientation to the transcription initiation control region.

Specific constructs, expression cassettes having in the 5' to 3' direction of transcription, a transcription and translation initiation control regulatory region comprising sequence immediately 5' to a structural gene preferentially expressed in plant seed during lipid accumulation, a DNA sequence encoding desaturase, and sequence 3' to the structural gene are also provided. The construct may preferably contain DNA sequences encoding plant desaturase obtainable (included obtained) from Carthamus, Rininus, Brassica or Simmondsia Δ-9 desaturase

genes. Transcription and translation initiation control regulatory regions are preferentially obtained from structural genes preferentially expressed in plant embryo tissue such as napin, seed-ACP or Bce-4.

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By this invention, methods and constructs to inhibit the production of endogenous desaturase are also provided. For example, an anti-sense construct comprising, in the 5' to 3' direction of transcription, a transcription initiation control regulatory region functional in a plant cell, and an anti-sense DNA sequence encoding a portion of a plant  $\Delta$ -9 desaturase may be integrated into a plant host cell to decrease desaturase levels.

In yet a different embodiment, this invention is directed to a method of producing plant desaturase in a host cell comprising the steps of growing a host cell comprising an expression cassette, which would contain in the direction of transcription, a) a transcription and translation initiation region functional in said host cell, b) the DNA sequence encoding a plant desaturase in reading frame with said initiation region, and c) and a transcript termination region functional in said host cell, under conditions which will promote the expression of the plant desaturase. Cells containing a plant desaturase as a result of the production of the plant desaturase encoding sequence and also contemplated herein.

By this invention, a method of modifying fatty acid composition in a host plant cell from a given level of fatty acid saturation to a different level of fatty acid saturation is provided by growing a host plant cell having integrated into its genome a recombinant DNA sequence encoding a plant desaturase in either a sense or anti-sense orientation under control of regulatory elements functional in said plant cell during lipid accumulation under conditions which will promote the activity of said regulatory elements. Plant cells having such a modified level of fatty acid saturation are also contemplated hereunder. Oilseeds having such a modified level of fatty

acid saturation and oils produced from such oilseeds are further provided.

# DETAILED DESCRIPTION OF THE INVENTION

5 A plant desaturase of this invention includes any sequence of amino acids, such as a protein, polypeptide, or peptide fragment, obtainable from a plant source which is capable of catalyzing the insertion of a first double bond into a fatty acyl-ACP moiety in a plant host cell, i.e., in 10 vivo, or in a plant cell-like environment, i.e. in vitro. "A plant cell-like environment" means that any necessary conditions are available in an environment (i.e., such factors as temperatures, pH, lack of inhibiting substances) which will permit the enzyme to function In particular, this invention relates to enzymes which add such a first 15 double bond at the ninth carbon position in a fatty acyl-ACP chain. There may be similar plant desaturase enzymes of this invention with different specificities, such as the  $\Delta$ -12 desaturase of carrot.

Nucleotide sequences encoding desaturases may be obtained from natural sources or be partially or wholly artificially synthesized. They may directly correspond to a desaturase endogenous to a natural plant source or contain modified amino acid sequences, such as sequences which have been mutated, truncated, increased or the like. Desaturases may be obtained by a variety of methods, including but not limited to, partial or homogenous purification of plant extracts, protein modeling, nucleic acid probes, antibody preparations and sequence

comparisons. Typically a plant desaturase will be derived in whole or in part from a natural plant source.

Of special interest are Δ-9 desaturases which are obtainable, including those with are obtained, from Cartharmus, Ricinus, Simmondsia, or Brassica, for example C. tinctorius, R. communis, S. chinensis and B. campestris, respectively, or from plant desaturases which are obtainable through the use of these sequences.

"Obtainable" refers to those desaturases which have

sufficiently similar sequences to that of the native sequences provided herein to provide a biologically active desaturase.

Once a DNA sequence which encodes a desaturase is obtained, it may be employed as a gene of interest in a nucleic acid construct or in probes in accordance with this invention. A desaturase may be produced in host cells for harvest or as a means of effecting a contact between the desaturase and its substrate. Constructs may be designed to produce desaturase in either prokaryotic or eukaryotic cells. Plant cells containing recombinant constructs encoding biologically active desaturase sequences, both expression and anti-sense constructs, as well as plants and cells containing modified levels of desaturase proteins are of special interest. For use in a plant cell, constructs may be designed which will effect an increase or a decrease in amount of endogenous desaturase available to a plant cell transformed with such a construct.

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Where the target gene encodes an enzyme, such as a 20 plant desaturase, which is already present in the host plant, there are inherent difficulties in analyzing mRNA, engineered protein or enzyme activity, and modified fatty acid composition or oil content in plant cells, especially in developing seeds; each of which can be evidence of 25 biological activity. This is because the levels of the message, enzyme and various fatty acid species are changing rapidly during the stage where measurements are often made, and thus it can be difficult to discriminate between changes brought about by the presence of the foreign gene and those brought about by natural developmental changes in the seed. Where an expressed  $\Delta$ -9 desaturase DNA sequence is derived from a plant species heterologous to the plant host into which the sequence is introduced and has a distinguishable DNA sequence, it is often possible to specifically probe for expression of the foreign gene with 35 oligonucleotides complimentary to unique sequences of the inserted DNA/RNA. And, if the foreign gene codes for a protein with slightly different protein sequence, it may be

possible to obtain antibodies which recognize unique epitopes on the engineered protein. Such antibodies can be obtained by mixing the antiserum to the foreign protein with extract from the host plant, or with extracts containing the host plant enzyme. For example, one can isolate antibodies uniquely specific to a C. tinctorius  $\Delta-$ 9 desaturase by mixing antiserum to the desaturase with an extract containing a Brassica  $\Delta$ -9 desaturase. approach will allow the detection of C. tinctorius desaturase in Brassica plants transformed with the C. 10 tinctorius desaturase gene. In plants expressing an endogenous gene in an antisense orientation, the problem is slightly different. In this case, there are no specific reagents to measure expression of a foreign protein. However, one is attempting to measure a decrease in an enzyme activity that normally is increasing during development. This makes detection of expression a simpler matter. In the final seed maturation phase, enzyme activities encoded by genes affecting oil composition usually disappear and cannot be detected in final mature seed. Analysis of the fatty acid content may be preformed

gas chromatography, for example. By increasing the amount of desaturase available in the plant cell, an increased percentage of unsaturated 25 fatty acids may be provided; by decreasing the amount of desaturase, an increased percentage of saturated fatty acids may be provided. (Modifications in the pool of fatty acids available for incorporation into triglycerides may likewise affect the composition of oils in the plant cell.) 30 Thus, an increased expression of desaturase in a plant cell may result in increased proportion of fatty acids, such as one or more of palmitoleate (C16:1), oleate (C18:1), linoleate (C18:2) and linolenate (C18:3) are expected. special interest is the production of triglycerides having increased levels of oleate. Using anti-sense technology, alternatively, a decrease in the amount of desaturase

available to the plant cell is expected, resulting in a

by any manner known to those skilled in the art, including

higher percentage of saturates such as one or more of laurate (C12:0), myristate (C14:0), palmitate (C16:0), stearate (C18:0), arachidate (C20:0), behemate (C22:0) and lignocerate (C24:0). Of special interest is the production of triglycerides having increased levels of stearate or palmitate and stearate. In addition, the production of a variety of ranges of such saturates is desired. Thus, plant cells having lower and higher levels of stearate fatty acids are contemplated. For example, fatty acid compositions, including oils, having a 10% level of stearate as well as compositions designed to have up to a 60% level of stearate or other such modified fatty acid(s) composition are contemplated.

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The modification of fatty acid compositions may also affect the fluidity of plant membranes. Different lipid concentrations have been observed in cold-hardened plants, for example. By this invention, one may be capable of introducing traits which will lend to chill tolerance. Constitutive or temperature inducible transcription initiation regulatory control regions may have special applications for such uses.

Other applications for use of cells or plants producing desaturase may also be found. For example, potential herbicidal agents selective for plant desaturase may be obtained through screening to ultimately provide environmentally safe herbicide products. The plant desaturase can also be used in conjunction with chloroplast lysates to enhance the production and/or modify the composition of the fatty acids prepared in vitro. The desaturase can also be used for studying the mechanism of fatty acid formation in plants and bacteria. For these applications, constitutive promoters may find the best use.

Constructs which contain elements to provide the transcription and translation of a nucleic acid sequence of interest in a host cell are "expression cassettes".

Depending upon the host, the regulatory regions will vary, including regions from structural genes from viruses, plasmid or chromosomal genes, or the like. For expression

in prokaryotic or eukaryotic microorganisms, particularly unicellular hosts, a wide variety of constitutive or regulatable promoters may be employed. Among transcriptional initiation regions which have been described are regions from bacterial and yeast hosts, such as E. coli, B. subtilis, Saccharomyces cerevisiae, including genes such as β-galactosidase, T7 polymerase, trp E and the like.

A recombinant construct for expression of desaturase in a plant cell ("expression cassette") will include, in 10 the 5' to 3' direction of transcription, a transcription and translation initiation control regulatory region (the transcriptional and translational initiation regions together often also known as a "promoter") functional in a plant cell, a nucleic acid sequence encoding a plant 15 desaturase, and a transcription termination region. Numerous transcription initiation regions are available which provide for a wide variety of constitutive or regulatable, e.g., inducible, transcription of the desaturase structural gene. Among transcriptional 20 initiation regions used for plants are such regions associated with cauliflower mosaic viruses (35S, 19S), and structural genes such as for nopaline synthase or mannopine synthase or napin and ACP promoters, etc. The transcription/translation initiation regions corresponding 25 to such structural genes are found immediately 5' upstream to the respective start codons. Thus, depending upon the intended use, different promoters may be desired.

Of special interest in this invention are the use of promoters which are capable of preferentially expressing the desaturase in seed tissue, in particular, at early stages of seed oil formation. Examples of such seed-specific promoters include the region immediately 5' upstream of napin or seed ACP genes, such as described in co-pending USSN 147,781, and the Bce-4 gene such as described in co-pending USSN 494,722. Alternatively, the use of the 5' regulatory region associated with an endogenous plant desaturase structural gene and/or the

transcription termination regions found immediately 3' downstream to the gene, may often be desired.

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In addition, for some applications, use of more than one promoter may be desired. For example, one may design a 5 dual promoter expression cassette each promoter having a desaturase sequence under its regulatory control. example, the combination of an ACP and napin cassette could be useful for increased production of desaturase in a seedspecific fashion over a longer period of time than either individually.

To decrease the amount of desaturase found in a plant host cell, anti-sense constructs may be prepared and then inserted into the plant cell. By "anti-sense" is meant a DNA sequence in the 5' to 3' direction of transcription in relation to the transcription initiation region, which encodes a sequence complementary to the sequence of a native desaturase. It is preferred that an anti-sense plant desaturase sequence be complementary to a plant desaturase gene indigenous to the plant host. Sequences found in an anti-sense orientation may be found in constructs providing for transcription or transcription and translation of the DNA sequence encoding the desaturase, including expression cassettes. Constructs having more than one desaturase sequence under the control of more than one promoter or transcription initiation region may also be employed with desaturase constructs. Various transcription initiation regions may be employed. One of ordinary skill in the art can readily determine suitable regulatory regions. Care may be necessary in selecting transcription initiation regions to avoid decreasing desaturase activity in plant cells other than oilseed tissues. Any transcription initiation region capable of directing expression in a plant host which causes initiation of adequate levels of transcription selectively in storage tissues during seed development for example, should be sufficient. As such, seed specific promoters may be desired. Other manners of decreasing the amount of endogenous plant desaturase, such as ribozymes or the

screening of plant cells transformed with constructs for rare events containing sense sequences which in fact act to decrease desaturase expression, are also contemplated herein. Other analogous methods may be applied by those of ordinary skill in the art.

By careful selection of plants, transformants having particular oils profiles may be obtained. This may in part depend upon the qualities of the transcription initiation region(s) employed or may be a result of culling transformation events to exploit the variabilities of expression observed.

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In order to obtain the nucleic acid sequences encoding C. tinctorius desaturase, a protein preparation free of a major albumin-type contaminant is required. As demonstrated more fully in the Examples, the protocols of 15 McKeon and Stumpf, supra, result in a preparation contaminated with a seed storage protein. Removal of the protein contaminant may be effected by application of a reverse-phase HPLC, or alternatively, by application of a reduction and alkylation step followed by electrophoresis 20 and blotting, for example. Other purification methods may be employed as well, now that the presence of the contaminant is confirmed and various properties thereof described. Once the purified desaturase is obtained it may be used to obtain the corresponding amino acid and/or 25 nucleic acid sequences thereto in accordance with methods familiar to those skilled in the art. Approximately 90% of the total amino acid sequence of the C. tinctorius desaturase is provided in Fig. 1 and in SEQ ID NOS: 1-11. The desaturase produced in accordance with the subject 30 invention can be used in preparing antibodies for assays for detecting plant desaturase from other sources.

A nucleic acid sequence of this invention may include genomic or cDNA sequence and mRNA. A cDNA sequence may or may not contain pre-processing sequences, such as transit peptide sequences. Transit peptide sequences facilitate the delivery of the protein to a given organelle and are

cleaved from the amino acid moiety upon entry into the organelle, releasing the "mature" sequence.

In Fig. 2 and SEQ ID NO: 13, the sequence of the C. tinctorius desaturase precursor protein is provided; both the transit peptide and mature protein sequence are shown. Also provided in this invention are cDNA sequences relating to R. communis desaturase (Fig. 3 and SEQ ID NOS: 14-15), B. campestris desaturase (Fig. 4 and SEQ ID NOS: 17-19) and S. chinesis (Fig. 5 and SEQ ID NOS: 43).

The use of the precursor cDNA sequence is preferred in desaturase expression cassettes. In addition, desaturase transit peptide sequences may be employed to translocate other proteins of interest to plastid organelles for a variety of uses, including the modulation of other enzymes related to the FAS pathway. See, European Patent Application Publication No. 189,707.

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As described in more detail below, the complete genomic sequence of a desaturase may be obtained by the screening of a genomic library with a desaturase cDNA probe and isolating those sequences which regulate expression in seed tissue. In this manner, the transcription, translation initiation regions and/or transcript termination regions of the desaturase may be obtained for use in a variety of DNA constructs, with or without the respective desaturase structural gene.

Other nucleic acid sequences "homologous" or "related" to DNA sequences encoding other desaturases are also provided. "Homologous" or "related" includes those nucleic acid sequences which are identical or conservatively substituted as compared to the exemplified C. tinctorius, R. communis, S. chinesis or B. campestris desaturase sequences of this invention or a plant desaturase which has in turn been obtained from a plant desaturase of this invention. By conservatively substituted is meant that codon substitutions encode the same amino acid, as a result of the degeneracy of the DNA code, or that a different amino acid having similar properties to the original amino acid is substituted. One skilled in the art will readily

(DNA and RNA) sequences encoding and the like may be prepared and used to screen and recover desaturase from other plant sources. Typically, nucleic acid probes are labeled to allow detection, preferably with radioactivity although enzymes or other methods may also be used. For immunological screening methods, antibody preparations either monoclonal or polyclonal are utilized. Polyclonal antibodies, although less specific, typically are more useful in gene isolation. For detection, the antibody is labeled using radioactivity or any one of a variety of second antibody/enzyme conjugate systems that are commercially available. Examples of some of the available antibody detection systems are described by Oberfilder (Focus (1989) BRL Life Technologies, Inc., 11:1-5).

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A "homologous" or "related" nucleic acid sequence will show at least about 60% homology, and more preferably at least about 70% homology, between the known desaturase sequence and the desired candidate plant desaturase of interest, excluding any deletions which may be present. Homology is determined upon comparison of sequence information, nucleic acid or amino acid, or through hybridization reactions. Amino acid sequences are considered homologous by as little as 25% sequence identity between the two complete mature proteins. (See generally, Doolittle, R.F., of URFS and ORFS, University Science Books, CA, 1986.)

Oligonucleotide probes can be considerably shorter than the entire sequence, but should be at least about 10, preferably at least about 15, more preferably at least 20 nucleotides in length. When shorter length regions are used for comparison, a higher degree of sequence identity is required than for longer sequences. Shorter probes are often particularly useful for polymerase chain reactions (PCR), especially when highly conserved sequences can be identified. (See, Gould, et al., PNAS USA (1989) 86:1934-1938.) Longer oligonucleotides are also useful, up to the full length of the gene encoding the polypeptide of

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interest. When longer nucleic acid fragments are employed (>100 bp) as probes, especially when using complete or large cDNA sequences, one would screen with low stringencies (for example 40-50°C below the melting temperature of the probe) in order to obtain signal from the target sample with 20-50% deviation, i.e., homologous sequences. (See, Beltz, et al., Methods in Enzymology (1983) 100:266-285.) Both DNA and RNA probes can be used.

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A genomic library prepared from the plant source of interest may be probed with conserved sequences from a known desaturase to identify homologously related sequences. Use of the entire cDNA may be employed if shorter probe sequences are not identified. Positive clones are then analyzed by restriction enzyme digestion and/or sequencing. When a genomic library is used, one or more sequences may be identified providing both the coding region, as well as the transcriptional regulatory elements of the desaturase gene from such plant source. In this general manner, one or more sequences may be identified providing both the coding region, as well as the transcriptional regulatory elements of the desaturase gene from such plant source.

In use, probes are typically labeled in a detectable manner (for example with 32P-labeled or biotinylated nucleotides) and are incubated with single-stranded DNA or RNA from the plant source in which the gene is sought, although unlabeled oligonucleotides are also useful. Hybridization is detected by means of the label after single-stranded and double-stranded (hybridized) DNA or DNA/RNA have been separated, typically using nitrocellulose paper or nylon membranes. Hybridization techniques suitable for use with oligonucleotides are well known to those skilled in the art. Thus, plant desaturase genes may be isolated by various techniques from any convenient plant. Plant desaturase of developing seed obtained from other oilseed plants, such as soybean, coconut, oilseed rape, sunflower, oil palm, peanut, cocoa, cotton, corn and the like are desired as well as from non-traditional oil

sources, including but not limited to spinach chloroplast, avocado mesocarp, cuphea, California Bay, cucumber, carrot, meadowfoam, Oenothera and Euglena gracillis.

Once the desired plant desaturase sequence is obtained, it may be manipulated in a variety of ways. 5 Where the sequence involves non-coding flanking regions, the flanking regions may be subjected to resection, mutagenesis, etc. Thus, transitions, transversions, deletions, and insertions may be performed on the naturally 10 occurring sequence. In addition, all or part of the sequence may be synthesized, where one or more codons may be modified to provide for a modified amino acid sequence, or one or more codon mutations may be introduced to provide for a convenient restriction site or other purpose involved with construction or expression. The structural gene may be further modified by employing synthetic adapters, linkers to introduce one or more convenient restriction sites, or the like.

Recombinant constructs containing a nucleic acid sequence encoding a desaturase of this invention may be 20 combined with other, i.e. "heterologous," DNA sequences in a variety of ways. By heterologous DNA sequences is meant any DNA sequence which is not naturally found joined to the native desaturase, including combinations of DNA sequences from the same plant of the plant desaturase which are not 25 naturally found joined together. In a preferred embodiment, the DNA sequence encoding a plant desaturase is combined in a DNA construct having, in the 5' to 3' direction of transcription, a transcription initiation control region capable of promoting transcription in a host 30 cell, and a DNA sequence encoding a desaturase in either a sense or anti-sense orientation. As described in more detail elsewhere, a variety of regulatory control regions containing transcriptional or transcriptional and translational regions may be employed, including all or part of the non-coding regions of the plant desaturase.

The open reading frame coding for the plant desaturase or functional fragment thereof will be joined at its 5' end

to a transcription initiation regulatory control region. In some instances, such as modulation of plant desaturase via a desaturase in an anti-sense orientation, a transcription initiation region or transcription/ translation initiation region may be used. In embodiments wherein the expression of the desaturase protein is desired in a plant host, a transcription/ translation initiation regulatory region, is needed. Additionally, modified promoters, i.e., having transcription initiation regions derived from one gene source and translation initiation regions derived from a different gene source or enhanced promoters, such as double 35S CaMV promoters, may be employed for some applications.

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As described above, of particular interest are those 5' upstream non-coding regions which are obtained from genes regulated during seed maturation, particularly those preferentially expressed in plant embryo tissue, such as ACP-and napin-derived transcription initiation control regions. Such regulatory regions are active during lipid accumulation and therefore offer potential for greater control and/or effectiveness to modify the production of plant desaturase and/or modification of the fatty acid Especially of interest are transcription composition. initiation regions which are preferentially expressed in seed tissue, i.e., which are undetectable in other plant parts. For this purpose, the transcript initiation region of acyl carrier protein isolated from B. campestris seed and designated as "Bcg 4-4" and an unidentified gene isolated from B. campestris seed and designated as "Bce-4" are also of substantial interest.

Briefly, Bce4 is found in immature embryo tissue at least as early as 11 days after anthesis (flowering), peaking about 6 to 8 days later or 17-19 days post-anthesis, and becoming undetectable by 35 days post-anthesis. The timing of expression of the Bce4 gene closely follows that of lipid accumulation in seed tissue. Bce4 is primarily detected in seed embryo tissue and to a lesser extent found in the seed coat. Bce4 has not been

detected in other plant tissues tested, root, stem and leaves.

Approximately 3.4 kb genomic sequence of Bce4 is provided in Fig. 8 and as SEQ ID NO: 27, including about 1 kb 5' to the structural gene, about 0.3 kb of the Bce4 coding gene sequence, and about 2.1 kb of the non-coding regulatory 3' sequence. Bce4 transcript initiation regions will contain at least 1 kb and more preferably about 5 to about 7.5 kb of sequence immediately 5' to the Bce4 structural gene.

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The Bcg 4-4 ACP message presents a similar expression profile to that of Bce4 and, therefore, also corresponds to lipid accumulation in the seed tissue. Bcg 4-4 is not found in the seed coat and may show some differences in expression level, as compared to Bce4, when the Bcg 4-4 5' non-coding sequence is used to regulate transcription or transcription and translation of a plant stearoyl-ACP desaturase of this invention. Genomic sequence of Bcg 4-4 is provided in Fig. 9 and as SEQ ID NO: 28, including about 1.5 kb 5' to the structural gene, about 1.2 kb of the Bcg 4-4 (ACP) structural gene sequence, and about 1.3 kb of the non-coding regulatory 3' sequence.

The napin 1-2 message is found in early seed development and thus, also offers regulatory regions which can offer preferential transcriptional regulation of a 25 desired DNA sequence of interest such as the plant desaturase DNA sequence of this invention during lipid accumulation. Napins are one of the two classes of storage proteins synthesized in developing Brassica embryos (Bhatty, et al., Can J. Biochem. (1968) 46:1191-1197) and 30 have been used to direct tissue-specific expression when reintroduced into the Brassica genome (Radke, et al., Theor. Appl. Genet. (1988) 75:685-694). Genomic sequence of mapin 1-2 is provided in Fig. 10 and as SEQ ID NO: 29, including about 1.7 kb 5' to the structural gene and about 35 1.3 kb of the non-coding regulatory 3' sequence

Regulatory transcript termination regions may be provided in DNA constructs of this invention as well.

Transcript termination regions may be provided by the DNA sequence encoding the plant desaturase or a convenient transcription termination region derived from a different gene source, especially the transcript termination region which is naturally associated with the transcript initiation region. The transcript termination region will contain at least about 1 kb, preferably about 3 kb of sequence 3' to the structural gene from which the termination region is derived.

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In developing the DNA construct, the various components of the construct or fragments thereof will normally be inserted into a convenient cloning vector which is capable of replication in a bacterial host, e.g., E. coli. Numerous vectors exist that have been described in the literature. After each cloning, the plasmid may be isolated and subjected to further manipulation, such as restriction, insertion of new fragments, ligation, deletion, insertion, resection, etc., so as to tailor the components of the desired sequence. Once the construct has been completed, it may then be transferred to an appropriate vector for further manipulation in accordance with the manner of transformation of the host cell.

Normally, included with the DNA construct will be a structural gene having the necessary regulatory regions for expression in a host and providing for selection of transformant cells. The gene may provide for resistance to a cytotoxic agent, e.g. antibiotic, heavy metal, toxin, etc., complementation providing prototrophy to an auxotrophic host, viral immunity or the like. Depending upon the number of different host species into which the expression construct or components thereof are introduced, one or more markers may be employed, where different conditions for selection are used for the different hosts.

The manner in which the DNA construct is introduced into the plant host is not critical to this invention. Any method which provides for efficient transformation may be employed. Various methods for plant cell transformation include the use of Ti- or Ri-plasmids, microinjection,

electroporation, liposome fusion, DNA bombardment or the like. In many instances, it will be desirable to have the construct bordered on one or both sides by T-DNA, particularly having the left and right borders, more particularly the right border. This is particularly useful when the construct uses A. tumefaciens or A. rhizogenes as a mode for transformation, although the T-DNA borders may find use with other modes of transformation.

Where Agrobacterium is used for plant cell

transformation, a vector may be used which may be
introduced into the Agrobacterium host for homologous
recombination with T-DNA or the Ti- or Ri-plasmid present
in the Agrobacterium host. The Ti- or Ri-plasmid
containing the T-DNA for recombination may be armed

(capable of causing gall formation) or disarmed (incapable
of causing gall formation), the latter being permissible,
so long as the vir genes are present in the transformed
Agrobacterium host. The armed plasmid can give a mixture
of normal plant cell and gall.

20 A preferred method for the use of Agrobacterium as the vehicle for transformation of plant cells employs a vector having a broad host range replication system, at least one T-DNA boundary and the DNA sequence or sequences of interest. Commonly used vectors include pRK2 or 25 derivatives thereof. See, for example, Ditta et al., PNAS USA, (1980) 77:7347-7351 and EPA 0 120 515, which are incorporated herein by reference. Normally, the vector will be free of genes coding for opines, oncogenes and virgenes. Included with the expression construct and the T-DNA will be one or more markers, which allow for selection of transformed Agrobacterium and transformed plant cells. A number of markers have been developed for use with plant cells, such as resistance to chloramphenicol, the aminoglycoside G418, hygromycin, or the like. 35 particular marker employed is not essential to this invention, one or another marker being preferred depending on the particular host and the manner of construction.

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The expression constructs may be employed with a wide variety of plant life, particularly plant life involved in the production of vegetable oils. These plants include, but are not limited to rapeseed, sunflower, C. tinctorius, cotton, Cuphea, peanut, soybean, oil palm and corn. Antisense constructs may be employed in such plants which share complementarity between the endogenous sequence and the antisense desaturase. Of special interest is the use of an antisense construct having a B. campestris desaturase in rapeseed, including B. campestris and B. napus.

For transformation of plant cells using Agrobacterium, explants may be combined and incubated with the transformed Agrobacterium for sufficient time for transformation, the bacteria killed, and the plant cells cultured in an appropriate selective medium. Once callus forms, shoot 15 formation can be encouraged by employing the appropriate plant hormones in accordance with known methods and the shoots transferred to rooting medium for regeneration of plants. The plants may then be grown to seed and the seed used to establish repetitive generations and for isolation 20 of vegetable oils compositions. A variety of stable genetic lines having fixed levels of saturation may be obtained and integrated into a traditional breeding program. Hemizygous and heterozygous lines or homozygous lines may demonstrate different useful properties for oil . 25 production and/or breeding. For example, saturation levels may be increased up to 2-fold by the development of homozygous plants as compared with heterozygous (including hemizygous) plants.

The invention now being generally described, it will be more readily understood by reference to the following examples which are included for purposes of illustration only and are not intended to limit the present invention.

EXAMPLES

### MATERIALS

Commercially available biological chemicals and chromatographic materials, including BSA, catalase (twice

crystalized from bovine liver), spinach ferredoxin, ferredoxin-NADP+ oxidoreductase (spinach leaf), NADPH, unlabeled fatty acids, DEAE-cellulose (Whatman DE-52) CNBractivated Sepharose 4B, and octyl-Sepharose, and Reactive Blue Agarose are from Sigma (St. Louis, MO).

Triethylamine, trichloroacetic acid, guanidine-HC1, and

Triethylamine, trichloroacetic acid, guanidine-HC1, and hydrazine-hydrate are also from Sigma. Proteolytic enzymes, including endoproteinases lysC, gluC, and aspN are sequencing grade enzymes obtained from Boehringer Mannheim

- (Indianapolis, IN). Organic solvents, including acetone, acetonitrile, methanol, ether and petroleum ether are purchased from J.T. Baker (Phillipsburg, NJ); concentrated acids and sodium sulfate are also from J.T. Baker (Phillipsburg, NJ). HPLC grade acetonitrile and
- trifluoracetic acid (TFA) are obtained from Burdick and Jackson (Muskegon, MI), and from Applied Biosystems (Foster City, CA), respectively. Radiochemicals, including [9,10(n)-3H] oleic acid (10mCi/µmol) and [3H]-iodoacetic acid (64Ci/mol) are from New England Nuclear (Boston, MA).
- Phenacyl-8 Reagent (bromoacetophenone with a crown ether catalyst) used to prepare phenacyl esters of the fatty acids for analysis are from Pierce (Rockford, IL). C18 reversed-phase thin-layer chromatography plates are from Whatman (Clifton, NJ).
- 25 Acyl carrier protein (ACP) and acyl-ACP synthase are isolated from E. coli strain K-12 as described by Rock and Cronan (Rock and Cronan, Methods in Enzymol (1981) 71:341-351 and Rock et al., Methods in Enzymol. (1981) 72:397-403). The E. coli is obtainable from Grain Processing (Iowa) as frozen late-logarithmic phase cells.

[9,10(n)- $^3$ H]stearic acid is synthesized by reduction of [9,10(n)- $^3$ H]oleic acid with hydrazine hydrate essentially as described by Johnson and Gurr (*Lipids* (1971) 6:78-84). [9,10(n)- $^3$ H]oleic acid (2 mCi), supplemented with 5.58mg unlabeled oleic acid to give a final specific radioactivity of 100mCi/mmol, is dissolved in 2ml of acetonitrile, acidified with 40 $\mu$ l of glacial acetic acid, and heated to 55°C. Reduction is initiated with 100 $\mu$ l of

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60% (w/w) hydrazine hydrate; oxygen is bubbled through the mixture continuously. After each hour acetonitrile is added to bring the volume back to 2ml and an additional 100µl of hydrazine hydrate is added. At the end of 5 hr. the reaction is stopped by addition of 3ml of 2M HCl. reaction products are extracted with three 3ml aliquots of petroleum ether and the combined ether extracts are washed with water, dried over sodium sulfate and evaporated to The dried reaction products are redissolved in 1.0ml acetonitrile and stored at -20°C. The distribution 10 of fatty acid products in a 15µl aliquot is determined by preparation of phenacyl esters, which are then analyzed by thin layer chromatography on C-18 reverse phase plates developed with methanol:water:95:5 (v/v). Usually reduction to [9,10(n)-3H] stearic acid is greater than 90%, 15 a small amount of unreacted oleic acid may remain. analysis is used to establish fraction of the total radioactivity that is present as stearate, and thereby to determine the exact substrate concentration in the enzyme 20 assay.

Acyl-ACP substrates, including [9,10(n)-3H] stearoyl-ACP are prepared and purified by the enzymatic synthesis procedure of Rock, Garwin, and Cronan (Methods in Enzymol. (1981) 72:397-403).

Acyl carrier protein was covalently bound to Sepharose 4B by reaction of highly purified ACP with CNBr-activated Sepharose 4B as described by McKeon and Stumpf (*J. Biol. Chem.* (1982) 257:12141-12147).

## 30 Example 1

In this example, an initial purification of *C*. tinctorius (safflower) desaturase, following the method of McKeon and Stumpf (*J. Biol. Chem.* (1982) 257:12141-12142), is described.

Assay: In each of the following steps, the presence of the enzyme is detected radiometrically by measuring enzyme-catalyzed release of tritium from [9,10(n)-

<sup>3</sup>H]stearoyl-ACP. Preparation of this substrate is described in "Materials" above.

The assay is performed by mixing 150µl water, 5ml dithiothreitol (100mM, freshly prepared in water),  $10\mu l$ bovine serum albumin (10mg/ml in water), 15 $\mu$ l NADPH (25mM, freshly prepared in 0.1M Tricine-HCl, pH 8.2), 25µl spinach ferredoxin (2mg/ml Sigma Type III in water),  $3\mu$ l NADPH: ferredoxin oxidoreductase (2.5 units/ml from Sigma), and 1  $\mu$ l bovine liver catalase (800,000 units/ml from Sigma); after 10 min at room temperature, this mixture is 10 added to a 13x100 mm screw-cap test tube containing 250µl sodium 1,4-piperazinediethanesulfonate (0.1M, pH 6.0). Finally,  $10\mu l$  of the sample to be assayed is added and the reaction is started by adding 30µl of the substrate, [9,10(n)- $^3$ H]stearoyl-ACP (100 $\mu$ Ci/ $\mu$ mol, 10 $\mu$ M in 0.1M sodium 1,4-piperazinediethanesulfonate, pH 5.8). After sealing with a cap, the reaction is allowed to proceed for 10 min. while shaking at 23°C. The reaction is terminated by addition of 1.2ml of 5.8% tricholoracetic acid and the resulting precipitated acyl-ACP's are removed by 20 centrifugation. The tritium released into the aqueous supernatant by the desaturase reaction is measured by liquid scintillation spectrometry. One unit of activity is defined as the amount of enzyme required to convert 1 mol of stearoyl-ACP to oleoyl-ACP, or to release  $4\mu g$ -atoms of 25 <sup>3</sup>H per minute.

Source tissue: Developing C. tinctorius seeds from greenhouse grown plants are harvested between 16 and 18 days after flowering, frozen in liquid nitrogen and stored at -70°C until extracted.

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Acetone Powder: Approximately 50g of frozen seeds are ground in liquid nitrogen and sieved to remove large seed coat pieces to provide a fine embryo powder. The powder is washed with acetone on a Buchner funnel until all yellow color is absent from the filtrate. The powder is then air dried and further processed as described below, or may be stored frozen for at least a year at -70°C without loss of enzyme activity.

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Acetone Powder Extract: The dried acetone powder is weighed and triturated with ten times its weight of 20mM potassium phosphate, pH 6.8; the mixture is then centrifuged at 12,000 x g for 20 minutes and decanted through a layer of Miracloth (Calbiochem, La Jolla, CA).

Ion Exchange Chromatography: The acetone powder extract is then applied to a DEAE-cellulose column (Whatman DE-52) (1.5 x 12 cm) equilibrated with 20mM potassium phosphate, pH 6.8. The pass-through and a wash with one column-volume (20ml) of buffer are pooled.

Affinity Chromatography: An affinity matrix for purification of the desaturase is prepared by reacting highly purified E. coli ACP, with CNBr-activated Sepharose 4B (Sigma). ACP (120mg) is reduced by treatment with 1mM dithiothreitol for 30 min on ice, and then desalted on Sephadex G-10 (Pharmacia) equilibrated with 0.1M sodium bicarbonate, pH 6.0. The treated ACP (20 ml, 6 mg/ml) is then mixed with 20ml of CNBr-activated Sepharose 4B swollen in 0.1M sodium bicarbonate, pH 7.0, and the mixture is allowed to stand at 4°C for one day. The gel suspension is then centrifuged, washed once with 0.1M sodium bicarbonate, pH 7.0, and then treated with 40ml 0.1M glycine, pH 8.0, for 4 hours at room temperature to block unreacted sites. The gel is then washed for five cycles with alternating 50ml volumes of 0.5M NaCl in 0.1M sodium acetate, pH 4.0, and 0.5M NaCl in 0.1M sodium bicarbonate, pH 6.5, to remove non-covalently bound ligand. The gel is loaded into a column (1.5 x 11.2 cm) and equilibrated in 20mM potassium phosphate, pH 6.8.

The combined fractions from the DE-52 column are applied to the column, which is subsequently washed with one column volume (20ml) of the equilibration buffer, and then with 2.5 column volumes (50ml) of 300mM potassium phosphate, pH 6.8. Fractions are assayed for protein using the BCA Protein Assay Reagent (Pierce, Rockford, IL) to make sure that all extraneous protein has been eluted. Active  $\Delta$ -9 desaturase is eluted from the column with 600mM potassium phosphate, pH 6.8. Active fractions are analyzed

by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) on 0.75mm thick 8 x 12 cm mini-gels according to the method of Laemmli (Nature (1970) 227:680). The running gel contains 10% acrylamide in a 30/0.8 ratio of acrylamide to cross-linker bis-acrylamide. Those fractions containing a predominant band at approximately 43 kD are pooled and stored frozen at -70°C until final purification. The yield from 50g of seed tissue is is approximately 60µg of protein as measured by amino acid analysis.

Further purification as described in Example 2 or Example 3 is then applied to the fractions pooled from the ACP-Sepharose column separation.

## 15 Example 2

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In this example, a protocol for the final purification of *C. tinctorius* desaturase is described. Seeds are treated in accordance with Example 1.

Reverse-Phase HPLC: Fractions from the ACP-Sepharose column are pooled and applied to a Vydac C4 reverse-phase column (0.45 x 15 cm) equilibrated in 0.1% TFA, 7% acetonitrile. After a 10 min wash with 0.1% TFA, the column is eluted with a gradient of increasing acetonitrile (7%-70% v/v) in 0.1% TFA over a period of 45 min. rate is 0.5ml/min throughout. Eluting components are monitored by absorbance at 214 nm.  $\Delta$ -9 desaturase elutes at about 42 min. (approximately 50% acetonitrile); the major contaminant protein remaining from ACP-affinity chromatography elutes at about 28 min. (approximately 30% acetonitrile). The substantially homogeneous desaturase, which is no longer active, is identified by SDS-PAGE, in which it exhibits a single band corresponding to a molecular weight of approximately 43 kD. The quantity of desaturase protein in the sample may be determined by amino acid analysis.

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#### Example 3

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In this example, a protocol for the final purification of C. tinctorius desaturase is described. Seeds are treated in accordance with Example 1.

Reduction and Alkylation: Protein is precipitated out of the pooled fraction solutions recovered from the ACP-Sepharose column with 10% (w/v) trichloroacetic acid, washed with cold (-20°C) acetone, and resuspended in 1 ml 500mM Tris-HCl, pH 8.6, containing 6M guanidine-HCl, 10mM EDTA, and 3.2 mM dithiothreitol. After 2 hours, 3.52 µmol  $[^3H]$ -iodoacetic acid (64 $\mu$ Ci/ $\mu$ mol, New England Nuclear) is added, and the reaction is allowed to proceed at room temperature in the dark for 2 hours, at which time the reaction is terminated by addition of 1µ1 (15µmol) 8mercaptoethanol. The sample is then re-precipitated with 10% (w/v) trichloroacetic acid, and the pellet again washed with cold (-20°C) acetone and resuspended in Laemmli's SDSsample buffer (Nature (1970) 227:680).

SDS-Polyacrylamide Gel Electrophoresis: The resulting 20 sample is boiled for 5 min. and then applied to a 1.5 mm thick, 8 x 12 cm, SDS-polyacrylamide mini-gel prepared as described by Laemmli, supra. The running gel contains 17.5% acrylamide in a 30:0.13 ratio of acrylamide to crosslinking bis-acrylamide. Separation is achieved by electrophoresis at 15 mA, for 2 hours at 4°C.

Blotting from SDS-gels to PVDF Membrane: Proteins are recovered from the gel by electroblotting at 5 mA/cm<sup>2</sup> to a four-layer sandwich of polyvinylidenedifluoride (PVDF) membrane for 2 h at 4°C in a buffer containing 10mM CAPS ("3-[cyclohexylamino]-1-propane-sulfonic acid"), pH 11. The membranes must be wetted in 50% methanol, prior to exposure to the blotting buffer. After blotting, the membrane layers are stained for 1-2 min. in 0.02% Coomassie Blue in 50% methanol, and then destained in 50% methanol. The desaturase is identified as a band corresponding to a molecular weight of about 43 kD; the major contaminant runs at or near the dye front of the gel corresponding to a molecular weight less than 20 kD.

The desaturase band on the PVDF membrane may be applied directly to the Edman sequencer (Applied Biosystems Model 477A) for determination of the N-terminal sequence of the intact protein, or for more extensive sequence

5 determination, may be eluted from the membrane in 40% acetonitrile to recover pure desaturase in solution. Acetonitrile is removed from the eluted desaturase by evaporation on a Speed-Vac (Savant; Farmingdale, NY), and the substantially homogeneous Δ-9 desaturase is resuspended in an appropriate buffer for subsequent proteolytic digestion as described in Example 4. The quantity of desaturase protein present may be determined by amino acid analysis.

Alternatively, if the sample is to be digested with trypsin or gluC protease to generate peptides for amino acid sequence analysis, proteins may be electroblotted to nitrocellulose membranes and stained with Ponceau or amido black.

## 20 Example 4

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In this example, a method for the determination of the amino acid sequence of a desaturase is described.

Reduction and Alkylation: Substantially homogenous stearoyl-ACP desaturase (See, Example 2) is reduced and alkylated with [3H]-iodacetic acid (See, Example 3), except that the final acetone-washed pellet is resuspended in the appropriate buffer for subsequent proteolysis. Reduction and alkylation assures complete denaturation of the protein so that complete proteolysis can occur. The sample may be alkylated with radiolabeled iodoacetamide or with 4-vinylpyridine instead of [3H]-iodacetic acid in substantially the same manner. Use of iodoacetic acid affords an alkylated sample with greater solubility, which is advantageous in subsequent sample manipulation.

Proteolysis: Substantially pure alkylated samples are digested with endoproteinase lysC. The sample is resuspended in 100 μl of 25 mM Tris-HCl, pH 8.8, containing 1 mM EDTA. Endoproteinase lysC is added to the sample in a

protease/desaturase ratio of 1/50 (w/w). Digestion is allowed to proceed at room temperature for 8 hours, at which time another equal amount of protease is added. After 18 more hours, 1 µl of concentrated HCl is added to stop proteolysis, and the sample is applied directly to a Vydac C18 reverse-phase column (0.2 x 15 cm) equilibrated in 7% acetonitrile (v/v) in 0.1 mM sodium phosphate, pH 2.2. After washing for 20 min with the equilibration buffer, peptides are eluted with a gradient in acetonitrile (7-70%, v/v) over 120 min. Flow rate is 50  $\mu$ l/min, throughout. Eluting components are monitored by absorbance at 214 nm, and individual peptide peaks are collected as separate fractions. The peptide fractions are further purified by application to a second Vydac C18 reverse-phase 15 column (0.2 x 15 cm) equilibrated in 7% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid. Again, after a 20 min wash with equilibration buffer, the substantially pure peptides are eluted with a gradient (7-70%, v/v) of acetonitrile in 0.1% trifluoroacetic acid over 120 min. The flow rate is 50  $\mu$ l/min, throughout. Eluting components 20 are monitored by absorbance at 214 nm, and individual peptide peaks are collected as separate fractions. These substantially pure peptides are applied directly to the Edman sequencer (Applied Biosystems, Model 477A) for amino acid sequence analysis. Alternatively, peptide fraction 25 from the first HPLC purification in phosphate buffer, or from a single chromatography step in trifluoroacetic acid buffer, may be applied directly to the sequencer, but these fractions, in many cases, are not substantially pure and yield mixed or ambiguous sequence information. 30

Other proteases may be used to digest desaturase, including but not limited to trypsin, gluC, and aspN. While the individual digest buffer conditions may be different, the protocols for digestion, peptide separation, purification, and sequencing are substantially the same as those outlined for the digestion with lysC. Alternatively, desaturase may be digested chemically using cyanogen bromide (Gross Methods Enzymol (1967) 11:238-255 or Gross

and Witkop J. Am. Chem. Soc. (1961) 83:1510), hydroxylamine (Bornstein and Balian Methods Enzymol. (1977) 47:132-745), iodosobenzoic acid (Inglis Methods Enzymol. (1983) 91:324-332), or mild acid (Fontana et al., Methods Enzymol. (1983) 91:311-317), as described in the respective references.

Fragments generated from these digestion steps of *C. tinctorius* desaturase are presented in Fig. 1 and as SEQ ID NOS: 1-11.

#### 10 Example 5

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In this example, the preparation of a plant embryo cDNA bank, using the methods as described in Alexander, et al. (Methods in Enzymology (1987) 154:41-64) and the screening of the bank to obtain a desaturase cDNA clone is described.

<u>C. tinctorius</u>: A plant embryo cDNA library may be constructed from poly(A) + RNA isolated from C. tinctorius embryos collected at 14-17 days post-anthesis. Poly(A) + RNA is isolated from polyribosomes by a method initially described by Jackson and Larkins (Plant Physiol. (1976) 57:5-10) as modified by Goldberg et al. (Developmental Biol. (1981) 83:201-217).

The plasmid cloning vector pCGN1703, derived from the commercial cloning vector Bluescribe M13- (Stratagene 25 Cloning Systems; San Diego, CA), is made as follows. The polylinker of Bluescribe M13- is altered by digestion with BamHI, treatment with mung bean endonuclease, and blunt-end ligation to create a BamHI-deleted plasmid, pCGN1700. pCGN1700 is digested with EcoRI and SstI (adjacent 30 restriction sites) and annealed with synthetic complementary oligonucleotides having the sequences 5' CGGATCCACTGCAGTCTAGAGGGCCCGGGA 3'(SEQ ID NO: 30) and 5' AATTTCCCGGGCCCTCTAGACTGCAGTGGATCCGAGCT 3' (SEQ ID NO: 31). These sequences are inserted to eliminate the EcoRI site, move the BamHI site onto the opposite side of the SstI (also, sometimes referred to as "SacI" herein) site found in Bluescribe, and to include new restriction sites PstI, XbaI, ApaI, SmaI. The resulting plasmid pCGN1702, is

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digested with *HindIII* and blunt-ended with Klenow enzyme; the linear DNA is partially digested with *PvuII* and ligated with T4 DNA ligase in dilute solution. A transformant having the *lac* promoter region deleted is selected (pCGN1703) and is used as the plasmid cloning vector.

Briefly, the cloning method for cDNA synthesis is as follows. The plasmid cloning vector is digested with SstI and homopolymer T-tails are generated on the resulting 3'overhang sticky-ends using terminal deoxynucleotidyl transferase. The tailed plasmid is separated from undigested or un-tailed plasmid by oligo(dA)-cellulose chromatography. The resultant vector serves as the primer for synthesis of cDNA first strands covalently attached to either end of the vector plasmid. The cDNA-mRNA-vector complexes are treated with terminal transferase in the presence of deoxyguanosine triphosphate, generating G-tails at the ends of the cDNA strands. The extra cDNA-mRNA complex, adjacent to the BamHI site, is removed by BamHI digestion, leaving a cDNA-mRNA-vector complex with a BamHI sticky-end at one end and a G-tail at the other. This complex is cyclized using the annealed synthetic cyclizing linker, 5'-

GATCCGCGGCCGCAATTCGAGCTCCCCCCCCC-3' and

#### 3'-GCGCCGGCGCTTAAGCTCGA-5'

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which has a BameI sticky-end and a C-tail end. Following ligation and repair the circular complexes are transformed into E. coli strain DH5α (BRL; Gaithersburg, MD) to generate the cDNA library. The C. tinctorius embryo cDNA bank contains between 3x10<sup>6</sup> and 5x10<sup>6</sup> clones with an average cDNA insert size of approximately 1000 base pairs.

Probe production Including PCR Reactions: Two regions of amino acid sequence (Example 4) with low codon degeneracy are chosen from opposite ends of peptide sequence "Fragment F2" (SEQ ID NO:2) for production of a probe for the plant desaturase cDNA. Two sets of mixed oligonucleotides are designed and synthesized for use as forward (SEQ ID NOS: 21-24) and reverse (SEQ ID NOS: 25-26) primers, respectively, in the polymerase chain reaction

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(Saiki et al., Science (1985) 230:1350-1354; Oste, Biotechniques (1988) 6:162-167). See, Fig. 6. oligonucleotides are synthesized on an Applied Biosystems 380A DNA synthesizer.

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Probes to C. tinctorius desaturase may be prepared using the peptide sequence "Fragment 2" (SEQ ID NO: 2) identified in Fig. 1. Four types of forward primers were synthesized and labeled 13-1, 13-2, 13-3, and 13-4 (SEQ ID NOS: 21-24, respectively). Two groups of reverse primers 10 were synthesized and designated 13-5A and 13-6A (SEQ ID NOS: 25-26, respectively). The primer sequences are shown in Fig. 6. These oligonucleotide groups have a redundancy of 64 or less and contain either 20 or 17 bases of coding sequence along with flanking restriction site sequences for HindIII or EcoRI. Based on the intervening amino acid sequence between the primer regions on peptide "Fragment 2" (SEQ ID NO: 2) the PCR product is expected to contain 107 base pairs.

Polymerase chain reaction is performed using the cDNA 20 library DNA as template and the possible eight combinations of the four forward and two reverse oligonucleotides as primers in a Perkin-Elmer/Cetus DNA Thermal Cycler (Norwalk, CT) thermocycle file 1 min. 94°C, 2 min. 42°C, 2 min rise from 42°-72°C for 30 cycles, followed by the step cycle file without step rises, 1 min. 94°C, 2 min. 42°C, 3 min. 72°C with increasing 15 sec extensions of the 72°C step for 10 cycles, and a final 10 min. 72°C extension.

The product of the 13-4 forward primer (SEQ ID NO: 24) and the 13-5A reverse primer (SEQ ID NO: 25) reaction was ethanol precipitated and then digested with HindIII and EcoRI, the resulting fragment was subcloned into pUC8 (Vieira and Messing, Gene (1982) 19:259-268). Minipreparation DNA (Maniatis et al., Molecular\_Cloning: A Laboratory Manual (1982) Cold Harbor Laboratory, New York) of one clone was sequenced by Sanger dideoxy sequencing (Sanger et al., Proc. Nat. Acad. Sci. USA (1977) 74:5463-5467) using the M13 universal and reverse primers. Translation of the resulting DNA sequence results in a

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peptide sequence that exactly matches the amino acid sequence in peptide "Fragment F2" (SEQ ID NO: 2).

An exact 50 base oligonucleotide designated DESAT-50 is synthesized to match the sequence of the PCR reaction product from the first valine residue to the last tyrosine residue.

# Library screen

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The C. tinctorius embryo cDNA bank is moved into the cloning vector lambda gt10 (Stratagene Cloning Systems) by digestion of total cDNA with EcoRI and ligation to lambda gt10 DNA digested with EcoRI. The titer of the resulting library was  $\sim 5 \times 10^5/\text{ml}$ . The library is then plated on E. coli strain C600 (Huynh, et al., DNA Cloning Vol. 1 Eds. Glover D.M. IRL Press Limited: Oxford England, pp. 56, 110) 20 at a density of 5000 plaques/150 mm NZY ("NZYM" as defined in Maniatis et al. supra) agar plate to provide over 45,000 plaques for screening. Duplicate lifts are taken of the plaques using NEN Colony Plaque Screen filters by laying precut filters over the plates for ~1 minute and then 25 peeling them off. The phage DNA is immobilized by floating the filters on denaturing solution (1.5M NaCl, .05M NaOH) for 1 min., transferring the filters to neutralizing solution (1.5M NaCl, 0.5M Tris-HCI pH 8.0) for 2 min. and then to 2XSSC (1xSSC = 0.15M NaCl; 0.015M Na citrate) for 3 30 min., followed by air drying. The filters are hybridized with <sup>32</sup>P end-labeled DSAT-50 oligonucleotide (SEQ ID NO: 32) (BRL 5' DNA Terminus Labeling System) by the method of Devlin et al., (DNA (1988) 7:499-807) at 42° C overnight, and washed for 30 min. at 50°C in 2XSSC, 0.5% SDS and then twice for 20 min. each at 50°C in 0.1XSSC, 0.5% SDS. 35 Filters are exposed to X-ray film at -70°C with a Dupont Cronex intensifying screen for 48 hours.

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Clones are detected by hybridization with the DSAT-50 oligonucleotide and plaque purified. The complete nucleotide sequence (SEQ ID NO: 12) of the cDNA insert of a clone, pCGN2754, and a partial restriction map thereof are presented in Figures 2 and 7A, respectively. The cDNA insert includes 1533 bases plus a poly(A) track at the 3' end of 100-200 bases. The open reading frame for the desaturase begins at the first ATG (nucleotide 106) from the 5' end and stops at nucleotide 1294. The translated amino acid sequence is presented in Fig. 2 and SEQ ID NO: 10 The open reading frame includes a 33 amino acid transit peptide not found in the amino acid sequence of the mature protein. The N-terminus of the protein begins at the alanine immediately following the NcoI site (nucleotide 202) indicating the site of the transit peptide processing. 15

#### Example 6

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In this example, expression of a plant desaturase in a prokaryote is described.

20 Desaturase expression construct in E. coli

A plasmid for expression of desaturase activity in *E. coli* is constructed as follows. The desaturase cDNA clone pCGN2754 is digested with *Hin*dIII and SalI and ligated to pCGN2016 (a chloramphenical resistant version of Bluescript KS-) digested with *Hin*dIII and *Xho*I. The resulting plasmid is pCGN1894.

pCGN2016 is prepared by digesting pCGN565 with HhaI, and the fragment containing the chloramphenicol resistance gene is excised, blunted by use of mung bean nuclease, and inserted into the EcoRV site of Bluescript KS- (Stratagene: La Jolla, CA) to create pCGN2008. The choramphenicol resistance gene of pCGN2008 is removed by EcoRI/HindIII digestion. After treatment with Klenow enzyme to blunt the ends, the fragment is ligated to DraI digested Bluescript KS-. A clone that has the DraI fragment containing ampicillin resistance replaced with the chloramphenicol resistance is chosen and named pCGN2016.

pCGN565 is a cloning vector based on pUC12-cm (K. Buckley Ph.D. Thesis, Regulation and expression of the phi X174 lysis gene, University of California, San Diego, 1985), but contains pUC18 linkers (Yanisch-Perron, et al., Gene (1985) 53:103-119).

The fragment containing the mature coding region of the Δ-9 desaturase, 3'-noncoding sequences and poly(A) tails is subcloned from pCGN1894 digested with Ncol and Asp718 into pUC120, an E. coli expression vector based on pUC118 (Vieira and Messing, Methods in Enzymology (1987) 153:3-11) with the lac region inserted in the opposite orientation and an Ncol site at the ATG of the lac peptide (Vieira, J. PhD. Thesis, University of Minnesota, 1988). The E. coli desaturase expression plasmid is designated pCGN3201. The desaturase sequences are inserted such that they are aligned with the lac transcription and translation signals.

# Expression of Desaturase in E.coli

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Single colonies of *E. coli* strain 7118 (Maniatis et al., supra) containing pUC120 or pCGN3201 are cultured in 80 mls each of ECLB broth, 300 mg/L penicillin. The cells are induced by the addition of 1mM IPTG. Cells are grown overnight (18 hrs) at 37° C.

Eighty mls of overnight cultures of E. coli (induced 25 and uninduced) containing pUC120 or pCGN3201 are centrifuged at 14,800 x g for 15 min. The pelleted cells are resuspended in 3 mls 20 mM phosphate buffer, pH 6.8. Resuspended cells were broken in a french press at 16,000 Broken cell mixtures are centrifuged 5000xg for 5 30 min. 100 µl of each supernatant is applied to a G-25 Sephadex gel filtration centrifugal column (Boehringer Mannheim Biochemicals), equilibrated in 20mM phosphate buffer pH 6.8. Columns are spun for 4 min at 5000xg. Effluent was collected and used as enzyme source in the 35 desaturase assay.

Desaturase activity is assayed as described in Example
1. Both pUC120-containing, IPTG-induced cells and
uninduced cells do not express detectable stearoyl-ACP

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desaturase activity. The pCGN3201 IPTG-induced extract contains 8.22 nmol/min of desaturase activity. pCGN3201 uninduced extracts contains 6.45 nmol/min of activity. The pCGN3201 IPTG-induced extract shows 21.5% more activity than the uninduced pCGN3201 extract.

Detection of induced protein in E. coli.

Extracts of overnight cultures of E. coli strain 7118 (Maniatis et al. supra ) containing pCGN3201 or pUC120 grown in ECLB containing 300 mg/L penicillin induced with 10 1mM IPTG are prepared as follows. 1.5 ml of overnight culture grown shaking at 37°C are pelleted in Eppendorf tubes for 10 min at 10-13,000 µg. Pellets are resuspended in 150 ul SDS sample buffer (0.05M Tris-HCl, pH6.8, 1% SDS, .5% B-mercaptoethanol, 10% glycerol and 0.005% bromophenol 15 blue) and boiled for 10 min. 25 µl of each sample are electrophoresed on a 10% polyacrylamide gel (Laemmli, Nature (1970) 227:680) at 25 mA for 5 hours. Gels are stained in 0.05% Coomassie Brilliant Blue, 25% isopropanol 20 and 10% acetic acid and destained in 10% acetic acid and 10% isopropanol. A band is detected at a position just below the 43,000 MW protein marker (SDS PAGE standard, Low molecular weight, BioRad, Richmond CA) in the pCGN3201 extracts that is not present in the pUC120 extracts. This 25 is the approximate molecular weight of mature desaturase protein.

# Requirement for Spinach Ferredoxin

Stearoyl-ACP desaturase can also be expressed in E.

coli by subcloning into the E. coli expression vector
pET8c (Studier, et al., Methods Enzymol. (1990) 185:60-89).

The mature coding region (plus an extra Met codon) of the
desaturase cDNA with accompanying 3'-sequences is inserted
as an Ncol - Sma 1 fragment into pET8c at the Ncol and

BamH1 sites (after treatment of the BamH1 site with Klenow
fragment of DNA polymerase to create a blunt end) to create
pCGN3208. The plasmid pCGN3208 is maintained in E. coli
strain BL21(DE3) which contains the T7 polymerase gene

under the control of the isopropyl-b-Dthiogalactopyranoside (IPTG)-inducible lacUV5 promoter (Studier et al., supra).

E. coli cells containing pCGN3208 are grown at 37°C to an OD595 of ~O.7 in NZY broth containing 0.4% (w/v) glucose and 300 mg/liter penicillin, and then induced for 3 hours with 0.4 mM IPTG. Cells are pelleted from 1 ml of culture, dissolved in 125  $\mu$ l of SDS sample buffer (10) and heated to 100°C for 10 min. Samples are analyzed by SDS

polyacrylamide gel electrophoresis at 25 mA for 5 h. Gels are stained in 0.05% Coomassie Brilliant Blue, 25% (v/v) isopropanol and 10% (v/v) acetic acid. A band is detected at a position just below the 43,000 MW protein marker (SDS PAGE standard, Low Molecular Weight, BioRad, Richmond, CA) in the pCGN3208 extract that is not present in the pET8c extracts. This is the approximate molecular weight of

mature desaturase protein.

For activity assays, cells are treated as described above and extracts are used as enzyme source in the stearoyl-ACP desaturase assay as described in Example 1. The extract from IPTG-induced pCGN3208 cells contains 8.61 nmol/min/mg protein of desaturase activity. The extract from pCGN3208 uninduced cells contains 1.41 nmol/min/mg protein of desaturase activity. The extract from pCGN3208 induced cells, thus has approximately 6-fold greater activity than the extract from uninduced pCGN3208 cells. Extracts from both induced and uninduced cells of pET8c do not contain detectable stearoyl-ACP desaturase activity.

Samples are also assayed as described in Example 1,

30 but without the addition of spinach ferredoxin, to
determine if the *E. coli* ferredoxin is an efficient
electron donor for the desaturase reaction. Minimal
activity is detected in *E. coli* extracts unless spinach
ferredoxin is added exogenously.

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# Example 7

In this example, the preparation of an ACP expression cassette containing a plant desaturase in a binary vector suitable for plant transformation is described.

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## ACP Expression Cassette

An expression cassette utilizing 5'-upstream sequences and 3'-downstream sequences obtainable from B. campestris ACP gene can be constructed as follows.

10 A 1.45kb XhoI fragment of Bcg 4-4 (Fig. 9 and SEQ ID NO: 28) containing 5'-upstream sequences is subcloned into the cloning/sequencing vector Bluescript+ (Stratagene Cloning Systems, San Diego, CA). The resulting construct, pCGN1941, is digested with XhoI and ligated to a 15 chloramphenicol resistant Bluescript M13+ vector, pCGN2015 digested with XhoI. pCGN2015 is prepared as described for pCGN2016 (See, Example 6) except that the EcoRI/HindIII "chloramphenicol" fragment isolated from pCGN2008 is ligated with the 2273 bp fragment of Bluescript KS+ 20 (Stratagene; LaJolla, CA) isolated after digestion with This alters the antibiotic resistance of the plasmid from penicillin resistance to chloramphenicol resistance. The chloramphenical resistant plasmid is pCGN1953.

3'-sequences of Bcg 4-4 are contained on an SstI/BglII 25 fragment cloned in the SstI/BamHI sites of M13 Bluescript+ vector. This plasmid is named pCGN1940. pCGN1940 is modified by in vitro site-directed mutagenesis (Adelman et al., DNA (1983) 2:183-193) using the synthetic oligonucleotide 5'-CTTAAGAAGTAACCCGGGCTGCAGTTTTAGTATTAAGAG-3' (SEQ ID NO: 33) to insert Smal and Pstl restriction sites immediately following the stop codon of the reading. frame for the ACP gene 18 nucleotides from the SstI site. The 3'-noncoding sequences from this modified plasmid, pCGN1950, are moved as a PsI-Smal fragment into pCGN1953 cut with PstI and SmaI. The resulting plasmid pCGN1977 comprises the ACP expression cassette with the unique restriction sites EcoRV, EcoRI and PstI available between the 1.45kb 5' and 1.5 kb of 3'-noncoding sequences (SEQ ID

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NO: 28) for the cloning of genes to be expressed under regulation of these ACP gene regions.

# Desaturase Expression in Plants

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Desaturase cDNA sequences from pCGN2754 are inserted 5 in the ACP expression cassette, pCGN1977, as follows. pCGN2754 is digested with HindIII (located 160 nucleotides upstream of the start codon) and Asp718 located in the polylinker outside the poly(A) tails. The fragment 10 containing the coding region for desaturase was blunt-ended using DNA polymerase I and ligated to pCGN1977 digested with EcoRV. A clone containing the desaturase sequences in the sense orientation with respect to the ACP promoter is selected and called pCGN1895. This expression cassette may be inserted into a binary vector, for example, for 15 Agrobacterium-mediated transformation, or employed in other plant transformation techniques.

# Binary Vector and Agrobacterium Transformation

The fragment containing the pCGN1895 expression sequences ACP 5'/desaturase/ACP 3' is cloned into a binary vector pCGN1557 (described below) for Agrobacterium transformation by digestion with Asp718 and XbaI and ligation to pCGN1557 digested with Asp718 and XbaI. The resulting binary vector is called pCGN1898.

pCGN1898 is transformed into Agrobacterium tumefaciens strain EHA101 (Hood, et al., J. Bacteriol. (1986) 168:1291-1301) as per the method of Holsters, et al., Mol. Gen. Genet. (1978) 163:181-187.

RNA blot analysis of seeds (T2) from T1 plants show the presence of a mRNA species for the inserted C. tinctorius desaturase, but the amount of message is low compared to endogenous levels of mRNA for the Brassica desaturase, suggesting that the expression levels were insufficient to significantly increase the amount of desaturase enzyme above that normally present. This is consistent with the negative results from oil, desaturase activity and Western blot analyses.

# Construction of pCGN1557

pCGN1557 (McBride and Summerfelt, Plant Molecular Biology (1990) 14(2):269-276) is a binary plant transformation vector containing the left and right T-DNA borders of Agrobacterium tumefaciens octopine Ti-plasmid pTiA6 (Currier and Nester, supra, the gentamycin resistance gene of pPH1JI (Hirsch and Beringer, supra), an Agrobacterium rhizogenes Ri plasmid origin of replication from pLJbB11 (Jouanin et al., supra), a 35S promoter-kanR-tml3' region capable of conferring kanamycin resistance to transformed plants, a ColE1 origin of replication from pBR322 (Bolivar et al., supra), and a lacZ' screenable marker gene from pUC18 (Yanish-Perron et al., supra).

There are three major intermediate constructs used to generate pCGN1557:

pCGN1532 (see below) contains the pCGN1557 backbone, the pRi plasmid origin of replication, and the ColE1 origin of replication.

pCGN1546 (see below) contains the CaMV35S5'-kanR-tml3' plant selectable marker region.

pCGN1541b (see below) contains the right and left T-DNA borders of the A. tumefaciens octopine Ti-plasmid and the lacz' region from pUC19.

To construct pCGN1557 from the above plasmids,
pCGN1546 is digested with XhoI, and the fragment containing
the CaMV 35S5'-kanR-tml3' region is cloned into the XhoI
site of pCGN1541b to give the plasmid pCGN1553, which
contains T-DNA/left border/CaMV 35S5'-kanR-tml3'/lacZ'/T
DNA left border. pCGN1553 is digested with BglII, and the
fragment containing the T-DNA/left border/CaMV35S5'-kanRtml3'/lacZ'/T-DNA left border region is ligated into BamHIdigested pCGN1532 to give the complete binary vector,
pCGN1557.

# 35 <u>pCGN1532</u>

The 3.5kb EcoRI-PstI fragment containing the gentamycin resistance gene is removed from pPh1JI (Hirsch and Beringer, Plasmid (1984) 12:139-141) by EcoRI-PstI

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digestion and cloned into EcoRI-PstI digested pUC9 (Vieira and Messing, Gene (1982) 19:259-268) to generate pCGN549. HindIII-PstI digestion of pCGN549 yields a 3.1 kb fragment bearing the gentamycin resistance gene, which is made blunt ended by the Klenow fragment of DNA polymerase I and cloned into PvuII digested pBR322 (Bolivar et al., Gene (1977) 2:95-113) to create pBR322Gm. pBR322Gm is digested with DraI and SphI, treated with Klenow enzyme to create blunt ends, and the 2.8 kb fragment cloned into the Ri origincontaining plasmid pLJbB11 (Jouanin et al., Mol. Gen. Genet. (1985) 201:370-374) which has been digested with Apal and made blunt-ended with Klenow enzyme, creating pLHbB11Gm. The extra ColE1 origin and the kanamycin resistance gene are deleted from pLHbB11Gm by digestion 15 with BamHI followed by self closure to create pGmB11. HindII site of pGmB11 is deleted by HindIII digestion followed by treatment with Klenow enzyme and self closure, creating pGmB11-H. The PstI site of pGmB11-H is deleted by PstI digestion followed by treatment with Klenow enzyme and self closure, creating pCGN1532.

# Construction of pCGN1546

The 35S promoter-tml3' expression cassette, pCGN986, contains a cauliflower mosaic virus 35S (CaMV35) promoter and a T-DNA tml 3'-region with multiple restriction sites 25 between them. pCGN986 is derived from another cassette, pCGN206, containing a CaMV35S promoter and a different 3' region, the CaMV region VI 3'-end. The CaMV 35S promoter is cloned as an AluI fragment (bp 7144-7734) (Gardner et. al., Nucl. Acids Res. (1981) 9:2871-2888) into the HincII site of M13mp7 (Messing, et. al., Nucl. Acids Res. (1981) 9:309-321) to create C614. An EcoRI digest of C614 produced the EcoRI fragment from C614 containing the 35S promoter which is cloned into the EcoRI site of pUC8 35 (Vieira and Messing, Gene (1982) 19:259) to produce pCGN147.

pCGN148a containing a promoter region, selectable marker (KAN with 2 ATG's) and 3' region, is prepared by

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digesting pCGN528 with BglII and inserting the BamHI-BglII promoter fragment from pCGN147. This fragment is cloned into the BglII site of pCGN528 so that the BglII site is proximal to the kanamycin gene of pCGN528.

5 The shuttle vector used for this construct pCGN528, is made as follows: pCGN525 is made by digesting a plasmid containing Tn5 which harbors a kanamycin gene (Jorgenson et. al., Mol. Gen. Genet. (1979) 177:65) with HindIII-BamHI and inserting the HindIII-BamHI fragment containing the 10 kanamycin gene into the HindIII-BamHI sites in the tetracycline gene of pACYC184 (Chang and Cohen, J. Bacteriol. (1978) 134:1141-1156). pCGN526 was made by inserting the BamHI fragment 19 of pTiA6 (Thomashow et. al., Cell (1980) 19:729-739), modified with XhoI linkers inserted into the SmaI site, into the BamHI site of 15 pCGN525. pCGN528 is obtained by deleting the small XhoI fragment from pCGN526 by digesting with XhoI and

pCGN149a is made by cloning the BamHI-kanamycin gene fragment from pMB9KanXXI into the BamHI site of pCGN148a. pMB9KanXXI is a pUC4K variant (Vieira and Messing, Gene (1982) 19:259-268) which has the XhoI site missing, but contains a functional kanamycin gene from Tn903 to allow for efficient selection in Agrobacterium.

religating.

25 pCGN149a is digested with HindIII and BamHI and ligated to pUC8 digested with HindIII and BamHI to produce pCGN169. This removes the Tn903 kanamycin marker. pCGN565 (see pCGN2016 description) and pCGN169 are both digested with HindIII and PstI and ligated to form pCGN203, a plasmid containing the CaMV 35S promoter and part of the .30 5'-end of the Tn5 kanamycin gene (up to the PstI site, Jorgenson et. al., (1979), supra). A 3'-regulatory region is added to pCGN203 from pCGN204 (an EcoRI fragment of CaMV (bp 408-6105) containing the region VI 3' cloned into pUC18 (Yanisch-Perron, et al., Gene (1985) 33:103-119) by 35 digestion with HindIII and PstI and ligation. resulting cassette, pCGN206, is the basis for the construction of pCGN986.

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The pTiA6 T-DNA tml 3'-sequences are subcloned from the Bam19 T-DNA fragment (Thomashow et al., (1980) supra) as a BamHI-EcoRI fragment (nucleotides 9062 to 12,823, numbering as in Barker et al., Plant Mol. Biol. (1982) 2:335-350) and combined with the pACYC184 (Chang and Cohen (1978), supra) origin of replication as an EcoRI-HindIII fragment and a gentamycin resistance marker (from plasmid pLB41), obtained from D. Figurski) as a BamHI-HindIII fragment to produce pCGN417.

The unique Smal site of pCGN417 (nucleotide 11,207 of 10 the Baml9 fragment) is changed to a SacI site using linkers and the BamHI-SacI fragment is subcloned into pCGN565 to give pCGN971. The BamHI site of pCGN971 is changed to an EcoRI site using linkers. The resulting EcoRI-SacI fragment containing the tml 3' regulatory sequences is joined to pCGN206 by digestion with EcoRI and SacI to give pCGN975. The small part of the Tn5 kanamycin resistance gene is deleted from the 3'-end of the CaMV 35S promoter by digestion with SalI and BglII, blunting the ends and ligation with SalI linkers. The final expression cassette 20 pCGN986 contains the CaMV 35S promoter followed by two SalI sites, an XbaI site, BamHI, SmaI, KpnI and the tml 3' region (nucleotides 11207-9023 of the T-DNA).

The 35S promoter-tml 3' expression cassette, pCGN986 is digested with HindIII. The ends are filled in with Klenow polymerase and XhoI linkers added. The resulting plasmid is called pCGN986X. The BamHI-SacI fragment of pBRX25 (see below) containing the nitrilase gene is inserted into BamHI-SacI digested pCGN986X yielding pBRX66.

Construction of pBRX25 is described in U.S. Letters Patent 4,810,648, which is hereby incorporated by reference. Briefly, the method is as follows: The nucleotide sequence of a 1212-bp PstI-HincII DNA segment encoding the bromoxynil-specific nitrilase contains 65-bp of 5' untranslated nucleotides. To facilitate removal of a portion of these excess nucleotides, plasmid pBRX9 is digested with PstI, and treated with nuclease Bal31. BamHI linkers are added to the resulting ends. BamHI-HincII

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fragments containing a functional bromoxynil gene are cloned into the BamHI-SmaI sites of pCGN565. The resulting plasmid, pBRX25, contains only 11 bp of 5' untranslated bacterial sequence.

pBRX66 is digested with PstI and EcoRI, blunt ends generated by treatment with Klenow polymerase, and XhoI linkers added. The resulting plasmid pBRX68 now has a tml 3' region that is approximately 1.1kb. pBRX68 is digested with SalI and SacI, blunt ends generated by treatment with Klenow polymerase and EcoRI linkers added. The resulting plasmid, pCGN986XE is a 35S promoter - tml 3' expression cassette lacking the nitrilase gene.

The Tn5 kanamycin resistance gene is then inserted into pCGN986XE. The 1.0 kb EcoRI fragment of pCGN1536 (see pCGN1547 description) is ligated into pCGN986XE digested with EcoRI. A clone with the Tn5 kanamycin resistance gene in the correct orientation for transcription and translation is chosen and called pCGN1537b. promoter  $Kan^R$ -tml 3' region is then transferred to a chloramphenical resistant plasmid backbone. pCGN786, (a pUC-CAM based vector with the synthetic oligonucleotide 5' GGAATTCGTCGACAGATCTCTGCAGCTCGAGGGATCCAAGCTT 3' (SEQ ID NO: 34) containing the cloning sites EcoRI, SalI, BglII, PstI, XhoI, BamHI, and HindIII inserted into pCGN566, pCGN566 contains the EcoHI-HindIII linker of pUC18 inserted into the EcoKI-HindIII sites of pUC13-cm (K. Buckler (1985) supra)) is digested with XhoI and the XhoI fragment of pCGN1537b containing the 35S promoter - KanR-tml 3' region is ligated in. The resulting clone is termed pCGN1546.

pCGN1541b

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pCGN565RBα2X (see below) is digested with BglII and XhoI, and the 728bp fragment containing the T-DNA right border piece and the lacZ' gene is ligated with BglII-XhoI digested pCGN65ΔKX-S+K (see below), replacing the BglII-XhoI right border fragment of pCGN65ΔKX-S+K. The resulting plasmid, pCGN65α2X contains both T-DNA borders and the lacZ' gene. The ClaI fragment of pCGN65α2X is

replaced with an XhoI site by digesting with ClaI blunting the ends using the Klenow fragment, and ligating with XhoI linker DNA, resulting in plasmid pCGN65α2XX. pCGN65α2XX is digested with BgIII and EcoRV, treated with the Klenow fragment of DNA polymerase I to create blunt ends, and ligated in the presence of BgIII linker DNA, resulting in pCGN65α2XX'. pCGN65α2XX' is digested with BgIII and ligated with BgIII digested pCGN1538 (see below), resulting in pCGN1541a, which contains both plasmid backbones.

10 pCGN1541a is digested with XhoI and religated. Ampicillin resistant, chlormaphenicol sensitive clones are chosen, which lack the pACYC184-derived backbone, creating pCGN1541b.

pCGN1538 is generated by digesting pBR322 with EcoRI and PvuII, treating with Klenow to generate blunt ends, and ligating with BglII linkers. pCGN1538 is ampicillin resistant, tetracycline sensitive.

## pCGN65∆KX-S+K

20 pCGN501 is constructed by cloning a 1.85 kb EcoRI-XhoI fragment of pTiA6 (Currier and Nester, J. Bact. (1976) 126:157-165) containing bases 13362-15208 (Barker et al., Plant Mo. Biol. (1983) 2:335-350) of the T-DNA (right border), into EcoRI-SalI digested Ml3mp9 (Vieira and 25 Messing, Gene (1982) 19:259-268). pCGN502 is constructed by cloning a 1.6 kb HindIII-SmaI fragment of pTiA6, containing bases 602-2212 of the T-DNA (left border), into HindIII-SmaI digested M13mp9. pCGN501 and pCGN502 are both digested with EcoRI and HindIII and both T-DNA-containing fragments cloned together into HindIII digested pUC9 30 (Vieira and Messing, Gene (1982) 19:259-268) to yield pCGN503, containing both T-DNA border fragments. pCGN503 is digested with HindIII and EcoRI and the two resulting HindIII-EcoRI fragments (containing the T-DNA borders) are 35 cloned into EcoRI digested pHC79 (Hohn and Collins, Gene (1980) 11:291-298) to generate pCGN518. The 1.6kb KpnI-EcoRI fragment from pCGN518, containing the left T-DNA border, is cloned into KpnI-EcoRI digested pCGN565 to

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generate pCGN580. The BamHII-BglII fragment of pCGN580 is cloned into the BamHI site of pACYC184 (Chang and Cohen, J. Bacteriol. (1978) 134:1141-1156) to create pCGN51. The 1.4 kb BamHI-SphI fragment of pCGN60 containing the T-DNA right border fragment, is cloned into BamHI-SphI digested pCGN51 to create pCGN65, which contains the right and left T-DNA borders.

pCGN65 is digested with KpnI and XbaI, treated with Klenow enzyme to create blunt ends, and ligated in the presence of synthetic BglII linker DNA to create pCGN65ΔKX. pCGN65ΔKX is digested with SalI, treated with Klenow enzyme to create blunt ends, and ligated in the presence of synthetic XhoI linker DNA to create pCGN65ΔKX-S+X.

# 15 pCGN565RBα2x

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pCGN451 (see below) is digested with HpaI and ligated in the presence of synthetic SphI linker DNA to generate pCGN55. The XhoI-SphI fragment of pCGN55 (bp13800-15208, including the right border, of Agrobacterium tumefaciens T-DNA; (Barker et al., Gene (1977) 2:95-113) is cloned into SalI-SphI digested pUC19 (Yanisch-Perron et al., Gene (1985) 53:103-119) to create pCGN60. The 1.4 kb HindIII-BamHI fragment of pCGN60 is cloned into HindIII-BamHI digested pSP64 (Promega, Inc.) to generate pCGN1039. pCGN1039 is digested with SmaI and NruI (deleting bp14273-15208; (Barker et al., Gene (1977) 2:95-113) and ligated in the presence of synthetic BglII linker DNA creating pCGN1039 $\Delta$ NS. The 0.47 kb *Eco*RI-HindIII fragment of pCGN1039 $\Delta$ NS is cloned into <code>EcoRI-HindIII</code> digested pCGN565 to create pCGN565RB. The HindIII site of pCGN565RB is replaced with an XhoI site by digesting with HindIII, treating with Klenow enzyme, and ligating in the presence of synthetic XhoI linker DNA to create pCGN565RB-H+X.

pUC18 (Norrander et al., Gene (1983) 26:101-106) is digested with HaeII to release the lacz' fragment, treated with Klenow enzyme to create blunt ends, and the lacz'-containing fragment ligated into pCGN565RB-H+X, which had been digested with AccI and SphI and treated with Klenow

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enzyme in such a orientation that the *lac*Z' promoter is proximal to the right border fragment; this construct, pCGN565RBα2x is positive for *lac*Z' expression when plated on an appropriate host and contains bp 13990-14273 of the right border fragment (Barker et al., Plant Mo. Biol. (1983) 2:335-350) having deleted the AccI-SphI fragment (bp 13800-13990).

## pCGN451

pCGN451 contains an ocs5'-ocs3' cassette, including
the T-DNA right border, cloned into a derivative of pUC8
(Vieira and Messing, supra). The modified vector is
derived by digesting pUC8 with HincII and ligating in the
presence of synthetic linker DNA, creating pCGN416, and
then deleting the EcoRI site of pCGN416 by EcoRI digestion
followed by treatment with Klenow enzyme and self-ligation
to create pCGN426.

The ocs5'-ocs3' cassette is created by a series of steps from DNA derived from the octopine Ti-plasmid pTiA6 (Currier and Nester, supra). To generate the 5'end, which includes the T-DNA right border, an EcoRI fragment of pTiA6 (bp 13362-16202 (the numbering is by Barker, et al., (Plant Mol. Bio (1983) 2:335-350) for the closely related Ti plasmid pTi15955)) is removed from pVK232 (Knauf and Nester, Plasmid (1982) 8:45) by EcoRI digestion and cloned into EcoRI digested pACYC184 (Chang and Cohen, supra) to generate pCGN15.

The 2.4kb BamHI-EcoRI fragment (bp 13774-16202) of pCGN15 is cloned into EcoRI-BamHI digested pBR322 (Bolivar, et al., supra) to yield pCGN429. The 412 bp EcoRI-BamHI fragment (bp 13362-13772) of pCGN15 is cloned into EcoRI-BamHI digested pBR322 to yield pCGN407. The cut-down promoter fragment is obtained by digesting pCGN407 with XmnI (bp 13512), followed by resection with Bal31 exonuclease, ligation of synthetic EcoRI linkers, and digestion with BamHI. Resulting fragments of approximately 130 bp are gel purified and cloned into M13mp9 (Vieira and Messing, supra) and sequenced. A clone, I-4, in which the EcoRI linker has been inserted at bp 1362 between the

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transcription initiation point and the translation initiation codon is identified by comparison with the sequence of de Greve, et al., (J. Mol. Appl. Genet. (1982) 1:499-512). The EcoRI cleavage site is at position 13639, downstream from the mRNA start site. The 141 bp EcoRI-BamHI fragment of I-4, containing the cut-down promoter, is cloned into EcoRI-BamHI digested pBR322 to create pCGN428. The 141 bp EcoRI-BamHI promoter piece from pCGN428, and the 2.5 kh EcoRI-BamHI ocs5' piece from pCGN429 are cloned together into EcoRI digested pUC19 (Vieira and Messing, supra) to generate pCGN442, reconstructing the ocs upstream region with a cut-down promoter section.

To generate the ocs3' end, the HindIII fragment of pLB41 (D. Figurski, UC San Diego) containing the gentamycin resistance gene is cloned into HindIII digested pACYC184 (Chang and Cohen, supra) to create pCGN413b. The 4.7 kb BamHI fragment of pTiA6 (supra), containing the ocs3' region, is cloned into BamHI digested pBR325 (F. Bolivar, Gene (1978) 4:121-136) to create 33c-19. The SmaI site at position 11207 (Barker, supra) of 33c-19 is converted to an XhoI site using a synthetic XhoI linker, generating pCCG401.2. The 3.8 kb BamHI-EcoRI fragment of pCGN401.2 is cloned into BamHI-EcoRI digested pCGN413b to create pCGN419.

The ocs5'-ocs3' cassette is generated by cloning the 2.64 kb EcoRI fragment of pCGN442, containing the 5' region, into EcoRI digested pCGN419 to create pCNG446. The 3.1kb XhoI fragment of pCGN446, having the ocs5' region (bp 13639-15208) and ocs3' region (bp 11207-12823), is cloned into the XhoI site of pCGN426 to create pCGN451.

## Example 8

In this example, the preparation of a Bce-4 expression cassette containing a plant desaturase is described.

35 The desaturase cDNA clone from pCGN2754 prepared as described in Example 5, is modified by in vitro mutagenesis to insert restriction sites immediately upstream of the ATG start codon and downstream of the TGA stop codon. A

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single-stranded template DNA is prepared for the mutagenesis reaction from pCGN1894 (described in Example 6) as described by Messing, (Methods in Enzymol. (1983) 101:20-79). Synthetic oligonucleotides are synthesized on an Applied Biosystems 380A DNA synthesizer. oligonucleotides used are

5'-CCATTTTGATCTTCCTCGAGCCCGGGCTGCAGTTCTTCTTCTTG-3'

(SEQ ID NO: 35) for the 5'mutagenesis and 5'-GCTCGTTTTTTTTTTCTCTGCAGCCCGGGCTCGAGTCACAGCTTCACC -3' (SEQ ID NO: 36) for the 3'-mutagenesis; both add PstI, SmaI 10 and XhoI sites flanking the coding region. Both oligonucleotides are 5'-phosphorylated (BRL 5'-Terminus labelling kit) and used for mutagenesis with the pCGN1894 template by the procedure of Adelman et al. (DNA (1983) 2:183-193). Alternatively, the desired restriction sites 15 may be inserted by PCR, using the 3' oligo described above (SEQ ID NO: 36) and another oligo, 5' ACTGACTGCAGCCCGGGCTCGAGGAAGATCAAAAATGGCTCTTC 3' (SEQ ID NO: 37) for the 3' and 5' primers, respectively. The 20 template in this polymerase chain reaction is DNA from pCGN1894. The XhoI fragment from the resulting clone can be subcloned into the Bce4 expression cassette, pCGN1870 (described below) at the unique XhoI site. Bce4/desaturase expression cassette can then be inserted in a suitable binary vector, transformed into Agrobacterium tumefaciens strain EHA101 and used to transform plants as

#### Bce-4 Expression Cassette

provided in Example 10.

pCGN1870 is a Bce-4 expression cassette containing 5' 30 and 3' regulatory regions of the Bce-4 gene and may be derived from the Bce-4 sequence found in pCGN1857, which was deposited with the ATCC on March 9, 1990, and assigned accession number 68251, or by methods known to one skilled in the art from the sequence (SEQ ID NO: 27) provided in 35 Fig. 8. The Bce 4 gene may be isolated as follows:

The ClaI fragment of pCGN1857, containing the Bce4 gene is ligated into ClaI digested Bluescript KS+

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(Stratagene; La Jolla, CA), producing pCGN1864. Single stranded DNA is made from pCGN1864 and altered by in vitro mutagenesis using the oligonucleotides

#### BCE45P:

5 (5'GAGTAGTGAACTTCATGGATCCTCGAGGTCTTGAAAACCTAGA3') (SEQ ID NO: 38) and

# BCE43P:

(5'CAATGTCTTGAGAGATCCCGGGATCCTTAACAACTAGGAAAAGG3') (SEQ ID NO: 39)

- as described by Adelman et al. (DNA (1983) 2:183-193). The oligonucleotide BSCP2 (5'GTAAGACACGACTTATCGCCACTG3') (SEQ ID NO: 40), complementary to a portion of Bluescript, is included in the reaction to improve the yield of double-stranded DNA molecules. The resulting plasmid, pCGN1866,
- contains XhoI and BamHI sites (from BCE45P) immediately 5' to the Bce4 start codon and BamHI and SmaI sites (from BCE43P) immediately 3' to the Bce4 stop codon. The ClaI fragment of pCGN1866, containing the mutagenized sequences, is inserted into the ClaI site of pCGN2016
- 20 (described in Example 6), producing pCGN1866C. The ClaI fragment of pCGN1866C is used to replace the corresponding wild-type ClaI fragment of PCGN1867 (described below) to produce pCGN1868. Bce4 coding sequences are removed by digestion of pCGN1868 with BamHI and recircularization of
- the plasmid to produce pCGN1870. The Bce4 expression cassette, pCGN1870, contains 7.4 kb of 5' regulatory sequence and 1.9 kb of 3' regulatory sequence derived from the Bce4 genomic clone separated by the cloning sites, XhoI, BamHI, and SmaI. Desaturase sequences in sense or
- anti-sense orientation may be inserted into the cassette via the cloning sites and the resulting construct may be employed in a plant transformation technique.

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#### pCGN1867

The BamHI and Smal sites of pUC18 are removed by BamHI-Smal digestion and recircularizing of the plasmid, without repair of the ends, to produce pCGN1862 The PstI fragment of pCGN1857, containing the Bce4 gene, is inserted into the PstI site of pCGN1862 to produce pCGN1867.

## Example 9

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In this example, the preparation of a napin 1-2 expression cassette containing a plant desaturase is described.

# Preparation of Desaturase Clone

The desaturase cDNA clone from pCGN2754 is prepared and modified as described in Example 8. The XhoI fragment from the resulting clone can be subcloned into the napin 1-2 expression cassette, pCGN1808 (described below) at the unique XhoI site. This napin 1-2/desaturase expression cassette can then be inserted into a suitable binary vector, transformed into A. tumefaciens strain EHA101 in a like manner as described in Example 7.

Alternatively, the desaturase safflower clone may be prepared such that restriction sites flank the translation start and stop sites, as described in Example 8, with the following modification. PCR was carried out according to manufacturer's instructions except for the initial annealing of the oligonucleotides to the template. reaction mix was heated to 90°C for 5 min, cooled to 37°C over a one hour period, kept at 37°C for 20 min and then subjected to standard PCR cycles. The PCR product was digested with PstI and ligated to pUC8 (Vieira and Messing (1982) Gene 19:2359-268) digested with PstI to produce pCGN3220. The NcoI/SacI fragment of pCGN3220 containing the pUC8 vector and the 5' and 3' sequences of the safflower desaturase cDNA was gel purified and ligated to the gel-purified cloned NcoI/SacI fragment from pCGN1894 (see Example 6). The resulting plasmid pCGN3222 contains safflower desaturase cDNA sequences partially from the cDNA WO 91/13972 55 PCT/US91/01746

clone and partially from the PCR. The regions obtained from the PCR were confirmed by DNA sequencing as being identical to the original cloned sequence.

# 5 Expression Cassettes Napin 1-2 pCGN1808 Expression Cassette

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An expression cassette utilizing 5' upstream sequences and 3' downstream sequences obtainable from B. campestris napin gene can be constructed as follows.

- 10 A 2.7 kb XhoI fragment of napin 1-2 (Fig. 10 and SEQ ID NO: 29) containing 5' upstream sequences is subcloned into pCGN789 (a pUC based vector the same as pUC119 with the normal polylinker replaced by the synthetic linker -5'GGAATTCGTCGACAGATCTCTGCAGCTCGAGGGATCCAAGCTT 3', SEQ ID 15 NO: 41, (which represented the polylinker EcoRI, SalI, BglII, PstI, XhoI, BamHI, HindIII) and results in pCGN940. The majority of the napin coding region of pCGN940 was deleted by digestion with SalI and religation to form pCGN1800. Single-stranded DNA from pCGN1800 was used in an 20 in vitro mutagenesis reaction (Adelman et al., DNA (1983) 2:183-193) using the synthetic oligonucleotide 5' GCTTGTTCGCCATGGATATCTTCTGTATGTTC 3', SEQ ID NO: 42. oligonucleotide inserted an EcoRV and an NcoI restriction site at the junction of the promoter region and the ATG start codon of the napin gene. An appropriate mutant was
  - A 1.7 kb promoter fragment was subcloned from pCGN1801 by partial digestion with EcoRV and ligation to pCGN786 (a pCGN566 chloramphenical based vector with the synthetic linker described above in place of the normal polylinker) cut with EcoRI and blunted by filling in with DNA Polymerase I Klenow fragment to create pCGN1802.

identified by hybridization to the oligonucleotide used for the mutagenesis and sequence analysis and named pCGN1801.

A 2.1 kb SalI fragment of napin 1-2 (Fig. 10 and SEQ

35 ID NO: 29) containing 3' downstream sequences is subcloned into pCGN789 (described above) and results in pCGN941. pCGN941 is digested with XhoI and HindIII and the resulting approximately 1.6 kb of napin 3' sequences are inserted

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into XhoI-HindIII digested pCGN1802 to result in pCGN1803. In order to remove a 326 nucleotide HindIII fragment inserted opposite to its natural orientation, as a result of the fact that there are 2 HindIII sites in pCGN1803, the pCGN1803 is digested with HindIII and religated. Following religation, a clone is selected which now contains only 1.25 kb of the original 1.6 napin 3' sequence. This clone, pCGN1808 is the napin 1-2 expression cassette and contains 1.725 kb of napin promoter sequences and 1.265 kb of napin 3' sequence with the unique cloning sites SalI, BgII, PstI and XhoI in between.

# Napin 1-2 pCGN3223 Expression Cassette

Alternatively, pCGN1808 may be modified to contain flanking restriction sites to allow movement of only the expression sequences and not the antibiotic resistance marker to binary vectors such as pCGN1557 (McBride and Summerfelt, supra). Synthetic oligonucleotides containing KpnI, NotI and HindIII restriction sites are annealed and ligated at the unique HindIII site of pCGN1808, such that only one HindIII site is recovered. The resulting plasmid, pCGN3200 contains unique HindIII, NotI and KpnI restriction sites at the 3'-end of the napin 3'-regulatory sequences as confirmed by sequence analysis.

25 The majority of the napin expression cassette is subcloned from pCGN3200 by digestion with HindIII and SacI and ligation to HindIII and SacI digested pIC19R (Marsh, et al. (1984) Gene 32:481-485) to make pCGN3212. The extreme 5'-sequences of the napin promoter region are reconstructed 30 by PCR using pCGN3200 as a template and two primers flanking the SacI site and the junction of the napin 5'promoter and the pUC backbone of pCGN3200 from the pCGN1808 construct. The forward primer contains ClaI, HindIII, NotI, and KpnI restiction sites as well as nucleotides 408-423 of the napin 5'-sequence (from the EcoRV site) and the reverse primer contains the complement to mapin sequences 718-739 which include the unique SacI site in the 5'promoter. The PCR was performed using a Perkin Elmer/Cetus

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thermocycler according to manufacturer's specifications. The PCR fragment is subcloned as a blunt-ended fragment into pUC8 (Vieira and Messing (1982) Gene 19:259-268) and digested with HincII to give pCGN3217. Sequence of pCGN3217 across the napin insert verifies that no improper nucleotides were introduced by PCR. The napin 5-sequences in pCGN3217 are ligated to the remainder of the napin expression cassette by digestion with ClaI and SacI and ligation to pCGN3212 digested with ClaI and SacI. resulting expression cassette pCGN3221, is digested with 10 HindIII and the napin expression sequences are gel purified away and ligated to pIC20H (Marsh, supra) digested with HindIII. The final expression cassette is pCGN3223, which contains in an ampicillin resistant background, essentially 15 identical 1.725 napin 5' and 1.265 3' regulatory sequences as found in pCGN1808. The regulatory regions are flanked with HindIII, NotI and KpnI restriction sites and unique SalI, BglII, PstI, and XhoI cloning sites are located between the 5' and 3' noncoding regions.

Desaturase sequences in sense or anti-sense orientation may be inserted into a napin expression cassette via the cloning sites. The resulting construct may be employed for plant transformation. For example, one of ordinary skill in the art could also use known techniques of gene cloning, mutations, insertion and repair to allow cloning of a napin expression cassette into any suitable binary vector, such as pCGN1557 (described in Example 7) or other similar vectors.

# 30 Desaturase Expression

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The coding region of the safflower desaturase contained in pCGN3222 is cloned into the pCGN3223 napin cassette by digestion with XhoI and ligation to pCGN3223 digested with XhoI and SalI. The resulting plasmid, pCGN3229 is digested with Asp718 and inserted in the binary vector pCGN1578 (McBride and Summerfelt (1990) Plant Mol. Biol. 14:269-276) at the unique Asp718 site. The resulting binary vector is pCGN3231 and contains the safflower

desaturase coding sequences flanked by the napin 5' and 3' regulatory sequences as well as the plant selectable marker construct, 35s/NPTII/tml.

The resulting binary vector, pCGN3231, is transformed into Agrobacterium and utilized for plant transformation as described in Example 10. For Northern analysis, total RNA is isolated from day 21 and day 28 post-anthesis developing seed from plants transformed with pCGN3231. Five samples were analyzed at day 21 and two at day 28 post-anthesis. RNA was isolated by the method of Hughes and Galau (Plant Mol. Biol. Reporter (1988) 6: 253-257). Northern blot analysis was performed using a labeled 0.8 kb BglII fragment of pCGN1894 as a probe. Prehybridization and hybridization was at 42°C in 50% formamide, 10X Denhardt's solution, 5X SSC, 0.1% SDS, 5mM EDTA and 100ug/ml denatured salmon sperm DNA. Filters were washed at 55°C in 0.1 X SSC, 0.1% SDS. Under these conditions, the probe does not hybridize to the endogenous Brassica desaturase gene sequences. mRNA complementary to the safflower desaturase was detected in all the transgenic samples examined. More mRNA was present at day 28 than at day 21 post-anthesis and the highest level of RNA was seen in transgenic 3231-8. The total safflower desaturase mRNA level was estimated to be ~0.01% of the message at day 28 post-anthesis.

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Western analysis (see below) gives a preliminary indication of increased protein in one transformant, 3231-8. However, the Western analysis is complicated by two factors: 1. The presence of cross-reacting material at the same molecular weight as expected for the safflower desaturase. We believe this material is the endogenous Brassica desaturase. 2. The analysis of levels of protein expressed is also complicated by the normal developmental increase in the expression of desaturase protein during this time period. If the seeds examined are not at the precise developmental stage as the control seeds, quantitative differences in the amount of material seen may be simply due to the normal increase in the Brassica

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desaturase over this time period and not due to the expression of the safflower desaturase.

#### Western Analysis

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Soluble protein is extracted from developing seeds of Brassica by homogenization with one volume (lml/gram fresh weight) of buffer containing 20mM potassium phosphate, pH 6.8. The homogenate is clarified by centrifugation at 12,000 x g for 10 minutes. A second centrifugation is performed if necessary to provide a non-particulate supernatant.

Protein concentration of the extract is measured by the micromethod of Bradford (Anal. Biochem. (1976) 72:248-254). Proteins (20-60µg) are separated by denaturing electrophoresis by the method of Laemmli (supra), and are transferred to nitrocellulose membrane by the method of Towbin et al. (Proc. Nat. Acad Sci. (1979) 76:4350-4354).

The nitrocellulose membrane is blocked by incubation at room temperature for 15 minutes or at 4°C overnight in Tris-buffered saline with Tween 20 (Polyoxyethylenesorbitan monolaurate) and "TTBS-milk", (TTBS = 20mM Tris-HC1, 500mM NaCl, 0.1% Tween 20 (v/v), pH 7.5; "TTBS-milk" = TTBS and 3% skim milk powder). The volume of liquid in all incubations with the nitrocellulose membrane is sufficient to cover the membrane completely. The membrane is then incubated for an additional 5 minutes in TTBS.

The nitrocellulose membrane is incubated for at least one hour with shaking at room temperature with rabbit antistearoyl-ACP desaturase antiserum that was diluted 5,000- or 10,000-fold in "TTBS-milk". The rabbit anti-desaturase antiserum was commercially prepared from desaturase protein (purified as described in Example 1) by Berkeley Antibody Co. (Richmond, CA). The membrane is washed twice by shaking with TTBS for 5 minutes and then with deionized  $\rm H_2O$  for 30 seconds.

The nitrocellulose membrane is incubated for at least 45 minutes at room temperature in a solution of "TTBS-milk" in which anti-rabbit IgG-alkaline phosphatase conjugate

(Promega, Madison, WI) is diluted 7,500-fold. The membrane is washed twice in TTBS followed by deionized  $\rm H_2O$ , as described above.

The nitrocellulose membrane is equilibrated in buffer containing 100mM Tris-HCl, 100mM NaCl, 50mM MgCl<sub>2</sub>, pH 9.5, by shaking for 5 minutes. The color reaction is initiated by placing the nitrocellulose membrane into 50ml of the same buffer to which has been added 15mg p-nitroblue tetrazolium chloride and 7.5mg 5-bromo- 4 chloro- 3-indolyl phosphate toluidine salt (BioRad Labs; Richmond, CA). The color reaction is stopped by rinsing the nitrocellulose membrane with deionized H<sub>2</sub>O and drying it between filter papers.

Oil analysis of developing seeds indicated no significant change in oil composition of the transformed plants with respect to the control plants. This result is consistant with the low levels of safflower mRNA observed in transgenic plants as compared to levels of endogenous Brassica desaturase (Example 12).

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#### Example 10

In this example, an Agrobacterium-mediated plant transformation is described. Brassica napus is exemplified. The method is also useful for transformation of other Brassica species including Brassica campestris.

## Plant Material and Transformation

Seeds of Brassica napus cv. Delta are soaked in 95% ethanol for 2 min, surface sterilized in a 1.0% solution of sodium hypochlorite containing a drop of Tween 20 for 45 min., and rinsed three times in sterile, distilled water. Seeds are then plated in Magenta boxes with 1/10th concentration of Murashige minimal organics medium (Gibco) supplemented with pyrodoxine (50  $\mu$ g/l), nicotinic acid (50  $\mu$ g/l), glycine (200  $\mu$ g/l), and 0.6% Phytagar (Gibco) pH 5.8. Seeds are germinated in a culture room at 22°C in a 16 h photoperiod with cool fluorescent and red light of

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intensity approximately 65  $\mu E$ insteins per square meter per second ( $\mu Em^{-2}S^{-1}$ ).

Hypocotyls are excised from 7 day old seedlings, cut into pieces approximately 4 mm in length, and plated on feeder plates (Horsch et al. 1985). Feeder plates are prepared one day before use by plating 1.0 ml of a tobacco suspension culture onto a petri plate (100x25 mm) containing about 30 ml MS salt base (Carolina Biological) 100 mg/l inositol, 1.3 mg/l thiamine-HCl, 200 mg KH2PO4 with 3% sucrose, 2,4-D (1.0 mg/l), 0.6% Phytagar, and pH adjusted to 5.8 prior to autoclaving (MSO/1/0 medium). sterile filter paper disc (Whatman 3 mm) is placed on top of the feeder layer prior to use. Tobacco suspension cultures are subcultured weekly by transfer of 10 ml of culture into 100 ml fresh MS medium as described for the feeder plates with 2,4-D (0.2 mg/l), Kinetin (0.1 mg/l). All hypocotyl explants are preincubated on feeder plates for 24 h. at 22°C in continuous light of intensity 30  $\mu\text{Em}^ ^{2}S^{-1}$  to 65 µEM $^{-2}S^{-1}$ .

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20 Single colonies of A. tumefaciens strain EHA101 containing a binary plasmid are transferred to 5 ml MG/Lbroth and grown overnight at 30°C. Per liter, MG/L broth contains 5g mannitol, 1 g L-glutamic acid or 1.15 g sodium glutamate, 0.25 g kH<sub>2</sub>PO<sub>4</sub>, 0.10 g NaCL, 0.10 g MGSO<sub>4</sub>·7H<sub>2</sub>O, 1 mg biotin, 5 g tryptone, and 2.5 g yeast extract, and the broth is adjusted to pH 7.0. Hypocotyl explants are immersed in 7-12 ml MG/L broth with bacteria diluted to  $1 \times 10^8$  bacteria/ml and after 10-20 min. are placed onto feeder plates. After 48 h of co-incubation with Agrobacterium, the hypocotyl explants are transferred to B5 30 0/1/0 callus induction medium which contains filter sterilized carbenicillin (500 mg/l, added after autoclaving) and kanamycin sulfate (Boehringer Mannheim) at concentrations of 25 mg/l.

After 3-7 days in culture at 65  $\mu \rm Em^{-2} \rm S^{-1}$  to 75  $\mu \rm Em^{-2} \rm S^{-1}$  continuous light, callus tissue is visible on the cut surface and the hypocotyl explants are transferred to shoot induction medium, B5BZ (B5 salts and vitamins supplemented

with 3 mg/l benzylaminopurine, 1 mg/l zeatin, 1% sucrose, 0.6% Phytagar and pH adjusted to 5.8). This medium also contains carbenicillin (500 mg/l) and kanamycin sulfate (25 mg/l). Hypocotyl explants are subcultured onto fresh shoot induction medium every two weeks.

Shoots regenerate from the hypocotyl calli after one to three months. Green shoots at least 1 cm tall are excised from the calli and placed on medium containing B5 salts and vitamins, 1% sucrose, carbenicillin (300 mg/l), kanamycin sulfate (50 mg/l) and 0.6% Phytagar) and placed in a culture room with conditions as described for seed germination. After 2-4 weeks shoots which remain green are cut at the base and transferred to Magenta boxes containing root induction medium (B5 salts and vitamins, 1% sucrose, 2 mg/l indolebutyric acid, 50 mg/l kanamycin sulfate and 0.6% Phytagar). Green rooted shoots are tested for NPT II activity.

#### Example 11

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In this example, a DNA-bombardment plant transformation is described. Peanut transformation is exemplified.

DNA sequences of interest may be introduced as expression cassettes, comprising at least a promoter region, a gene of interest, and a termination region, into a plant genome via particle bombardment as described in European Patent Application 332 855 and in co-pending application USSN 07/225,332, filed July 27, 1988.

Briefly, tungsten or gold particles of a size ranging from 0.5µM-3µM are coated with DNA of an expression cassette. This DNA may be in the form of an aqueous mixture or a dry DNA/particle precipitate.

Tissue used as the target for bombardment may be from cotyledonary explants, shoot meristems, immature leaflets, or anthers.

The bombardment of the tissue with the DNA-coated particles is carried out using a Biolistics™ particle gun (Dupont; Wilmington, DE). The particles are placed in the barrel at variable distances ranging from 1cm-14cm from the

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barrel mouth. The tissue to be bombarded is placed beneath the stopping plate; testing is performed on the tissue at distances up to 20 cm. At the moment of discharge, the tissue is protected by a nylon net or a combination of nylon nets with mesh ranging from 10µM to 300µM.

Following bombardment, plants may be regenerated following the method of Atreya, et al., (Plant Science Letters (1984) 34:379-383). Briefly, embryo axis tissue or cotyledon segments are placed on MS medium (Murashige and Skoog, Physio. Plant. (1962) 15:473) (MS plus 2.0 mg.l 6-benzyladenine (BA) for the cotyledon segments) and incubated in the dark for 1 week at 25 ± 2°C and are subsequently transferred to continuous cool white fluorescent light (6.8 W/m²). On the 10th day of culture, the plantlets are transferred to pots containing sterile soil, are kept in the shade for 3-5 days are and finally moved to greenhouse.

The putative transgenic shoots are rooted.

Integration of exogenous DNA into the plant genome may be confirmed by various methods known to those skilled in the art.

## Example 12

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This example describes methods to obtain desaturase 25 cDNA clones from other plant species using the DNA from the C. tinctorius  $\Delta$ -9 desaturase clone as the probe.

Isolation of RNA for Northern Analysis
Poly(A) + RNA is isolated from C. tinctorius embryos
collected at 14-17 days post-anthesis and Simmondsia
chinensis embryos as described in Example 5.

Total RNA is isolated from days 17-18 days post-anthesis Brassica campestris embryos by an RNA minipreparation technique (Scherer and Knauf, Plant Mol. Biol. (1987) 9:127-134). Total RNA is isolated from R. communis immature endosperm of about 14-21 days post-anthesis by a method described by Halling, et al. (Nucl. Acids Res. (1985) 13:8019-8033). Total RNA is isolated

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from 10 g each of young leaves from B. campestris, B. napus, and C. tinctorius, by extraction of each sample in 5 ml/g tissue of 4 M guanidine thiocyanate buffer as described by Colbert et al. (Proc. Nat. Acac. Sci. (1983) 80:2248-2252). Total RNA is also isolated from immature embryos of Cuphea hookeriana by extraction as above in 10 ml/g tissue.

Total RNA is isolated from immature embryos of California bay (Umbellularia californica) by an adaptation of the method of Lagrimini et al. (Proc. Nat. Acad. Sci. (1987) 84:7542-7546). Following homogenization in grinding buffer (2.5 ml/g tissue) as described, RNA is precipitated from the aqueous phase by addition of 1/10 volume 3 M sodium acetate and 2 volumes ethanol, followed by freezing at  $-80^{\circ}$ C for 30 minutes and centrifugation at 13,000 x g for 20 minutes. The pellets are washed with 80% ethanol and centrifugation is repeated as above. The pellets are resuspended in water, two volumes of 4 M LiCl are added, and the samples are placed at -20°C overnight. Samples are centrifuged as above and the pellets washed with 80% ethanol. Ethanol precipitation is repeated as above.

Total RNA is further purified from B. campestris, B. napus, and C. tinctorius leaves, and from C. tinctorius, B. campestris, California bay, and jojoba, and from R. communis immature endosperm, by removing polysaccharides on a 0.25 g Sigma Cell 50 cellulose column. The RNA is loaded onto the column in 1 ml of loading buffer (20 mM Tris-HCl pH 7.5, 0.5M NaCl, 1mM EDTA, 0.1% SDS), eluted with loading buffer, and collected in 500 µl fractions. Ethanol is 30 added to the samples to precipitate the RNA. The samples are centrifuged, and the pellets resuspended in sterile distilled water, pooled, and again precipitated in ethanol. The sample is centrifuged, and the resulting RNA is subjected to oligo(dT)-cellulose chromatography to enrich for poly(A) + RNA as described by Maniatis et al. (Molecular 35 Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1982)). Poly(A) + RNA is also

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purified from total *Cuphea hookeriana* RNA by oligo(dT)-cellulose chromatography.

Northern Analysis Using C. tinctorius Desaturase Clone: 2.5 µg of poly(A) + RNA from each of the above described poly(A) + samples from immature embryos of jojoba, Cuphea hookeriana, California bay, Brassica campestris, and C. tinctorius, from immature endosperm of R. communis, and from leaves of C. tinctorius, B. campestris, and B. napus are electrophoresed on formaldehyde/agarose gels (Fourney et al., Focus (1988) 10:5-7) and transferred to a Hybond-C 10 extra (Amersham, Arlington Heights, IL) filter according to manufacturer's specifications. The filter is prehybridized for four hours and hybridized overnight at 42°C in a roller bottle containing 10 ml of hybridization buffer (1 M NaCl, 1% SDS, 50% formamide, 0.1 mg/ml denatured salmon sperm 15 DNA) in a Hybridization Incubator, model 1040-00-1 (Robbins Scientific Corporation, Sunnyvale, CA). The probe used in the hybridization is a gel-isolated BglII fragment of the  $\Delta$ -9 desaturase clone that is labeled with  $^{32}P$ -dCTP using a BRL (Gaithersburg, MD) nick-translation kit, following 20 manufacturer's instructions. The blot is washed three times for 20 minutes each in 2X SSC, 0.5% SDS at 55°C. blot is exposed at -80°C, with a Dupont Cronex intensifying screen, to X-ray film for four days.

The autoradiograph shows that the *C. tinctorius* desaturase gene is expressed in both immature embryos and leaves of *C. tinctorius*, although the level of expression is considerably higher in embryos than in leaves. The autoradiograph also shows hybridization of the *C. tinctorius* desaturase clone to mRNA bands of a similar size in immature embryos from jojoba and California bay, and immature endosperm from *R. communis*. Hybridization is also detectable in RNA from *B. campestris* embryos upon longer exposure of the filter to X-ray film.

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R. communis cDNA Library Construction: A plant seed cDNA library may be constructed from poly(A) + RNA isolated from R. communis immature endosperm as described above.

The plasmid cloning vector pCGN1703, and cloning method are

as described in Example 5. The R. communis endosperm cDNA bank contains approximately  $2 \times 10^6$  clones with an average cDNA insert size of approximately 1000 base pairs.

The R. communis immature endosperm cDNA bank is moved into the cloning vector lambda gt22 (Stratagene Cloning Systems) by digestion of total cDNA with NotI and ligation to lambda gt22 DNA digested with NotI. The resulting phage are packaged using a commercially available kit and titered using E. coli strain LE392 (Stratagene Cloning Systems, La Jolla, CA). The titer of the resulting library was approximately 1.5 x 10<sup>7</sup> pfu/ml.

R. communis cDNA Library Screen: The library is plated on E. coli strain LE392 at a density of approximately 25,000 pfu/150mm NZY plate to provide approximately 50,000 plaques for screening. Phage are 15 lifted in duplicate on to NEN (Boston, MA.) Colony/Plaque Screen filters as described in Example 5. Following prehybridization at 42°C in 25 ml of hybridization buffer (1 M NaCl, 1% SDS, 50% formamide, 0.1 mg/ml denatured salmon sperm DNA) filters are hybridized overnight with a 20 gel-purified 520 base pair BglII fragment of the C. tinctorius desaturase clone (Figure 7A) that is radiolabeled with  $^{32}P$ -dCTP using a BRL (Gaithersburg, MD) Nick Translation System. Filters are washed three times 25 for 20 minutes each in 2X SSC, 0.5% SDS at 55°C in a shaking water bath. Filters are exposed to X-ray film overnight at -80°C with a Dupont Cronex intensifying screen.

Clones are detected by hybridization on duplicate

filters with the *C. tinctorius* desaturase cDNA fragment and
plaque purified. During plaque purification, it was
observed that larger plaques were obtained when *E. coli*strain Y1090 (Young, R.A. and Davis, R.W., Proc. Natl.
Acad. Sci. USA (1983) 80:1194) was used as the host

strain. This strain was thus used in subsequent plaque
purification steps. Phage DNA is prepared from the
purified clones as described by Grossberger (NAR (1987)
15:6737) with the following modification. The proteinase K

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treatment is replaced by the addition of 10% SDS and a 10 minute incubation at room temperature. Recovered phage DNA is digested with EcoRI, religated at low concentration, and transformed into E. coli DH5 $\alpha$ (BRL; Gaithersburg, MD) cells to recover plasmids containing cDNA inserts in pCGN1703. Minipreparation DNA (Maniatis et al., supra) is prepared from the clones and DNA sequence is determined as described above. Partial nucleotide sequence of the cDNA insert of a R. communis desaturase clone pCGN3230 is presented in

Figure 3A and SEQ ID NO: 14. The complete nucleotide sequence of this clone is presented in Fig. 3B and as SEQ ID NO: 15.

Northern Analysis Using R. communis Desaturase Clone:
Total RNA for Northern analysis is isolated from tobacco
leaves by the method of Ursin et al. (Plant Cell (1989)
1:727-736), petunia and tomato leaves by the method of
Ecker and Davis (Proc.Nat.Acad.Sci. (1987) 84:5202-5206),
and corn leaves by the method of Turpen and Griffith
(Biotechniques (1986) 4:11-15). Total RNA samples from
tobacco, corn, and tomato leaves are enriched for poly(A)+
RNA by oligo(dT)-cellulose chromatography as described by
Maniatis et al. (supra).

Poly(A) + RNA samples from tomato leaves (4  $\mu$ g) and corn and tobacco leaves (1 µg each), and total RNA from petunia leaves (25  $\mu$ g) are electrophoresed on a 25 formaldehyde/agarose gel as described by Shewmaker et al. (Virology (1985) 140:281-288). Also electrophoresed on this gel are poly(A) + RNA samples isolated from B. campestris day 17-19 embryos and B. campestris leaves (2 µg each), immature embryos from C. tinctorius, bay, and jojoba (1  $\mu$ g each), and R. communis endosperm (1  $\mu$ g). isolation of these poly(A) + RNA samples is described above for the Northern analysis using C. tinctorius desaturase cDNA as probe. The RNA is transferred to a nitrocellulose filter as described by Shewmaker et al. (supra) and 35 prehybridized and hybridized at 42°C in 50% formamide, 10X Denhardt's solution (described in Maniatis et al. (supra)), 5X SSC, 0.1% SDS, 5 mM EDTA, 100 ug/ml denatured salmon

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sperm DNA, and 10% dextran sulfate (in hybridization buffer only). The probe for hybridization is the <sup>32</sup>p-labeled (BRL Nick Translation Kit) 1.7 kb SalI insert of pCGN3230 that has been gel-purified from minipreparation DNA. The filter is washed following hybridization for 30 minutes in 2X SSC, 0.1% SDS at 42°C and at 50°C twice for 15 minutes each. The filter is exposed to X-ray film overnight at -80°C with a Dupont Cronex intensifying screen.

The autoradiograph shows hybridization of the R. communis desaturase clone to mRNA bands of a similar size in immature embryos from B. campestris, California bay, and C. tinctorius, and also in corn leaves and R. communis endosperm.

B. campestris Embryo cDNA Library Construction: RNA is isolated from 5 g of B. campestris cv. R500 embryos obtained from seeds harvested at days 17-19 post-anthesis. RNA is extracted in 25 mls of 4 M guanidine thiocyanate buffer as described by Colbert et al. (PNAS (1983) 80:2248-2252). Polysaccharides are removed from the RNA sample by resuspending the pellet in 6 ml of 1X TE (10 mM Tris/1 mM EDTA pH 8), adding potassium acetate to a concentration of 0.05M, and adding one half volume of ethanol. The sample is placed on ice for 60 minutes and centrifuged for 10 minutes at 3000 x q. RNA is precipitated from the supernatant by adding sodium acetate to a concentration of 0.3 M followed by the addition of two volumes of ethanol. RNA is recovered from the sample by centrifugation at 12,000 x g for 10 minutes and yield calculated by UV spectrophotometry. mg of the total RNA is further purified by removing polysaccharides on a 0.25 g Sigma Cell 50 cellulose column, as described above, and is also enriched for poly(A) + RNA by oligo(dT)-cellulose chromatography as described above.

A B. campestris day 17-19 post anthesis embryo cDNA library is constructed in plasmid vector pCGN1703 as described in Example 5, using 5 ug of the above described poly(A) + RNA. The library, which consists of approximately 1.5 x 10<sup>5</sup> transformants, is amplified by plating and scraping colonies, and is stored as frozen E. coli cells in

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10% DMSO at -80° C. DNA is isolated from a portion of the amplified library by scaling up the alkaline lysis technique of Birnboim and Doly (Nucleic Acids Res. (1979) 7:1513), and purified by CsCl centrifugation. Library DNA is digested with EcoRI and is cloned into EcoRI-digested bacteriophage lambda gt10 (Stratagene; La Jolla, CA) DNA. The DNA is packaged using Gigapack II Gold in vitro packaging extracts (Stratagene; La Jolla, CA) according to manufacturer's specifications. The titer of the phage stock, determined by dilution plating of phage in E. coli C600 hfl- cells (Huynh, et al., DNA Cloning. Volume 1. Eds. Gover, D.M. (1985) IRL Press Limited: Oxford, England, pp. 56,110), is 6 x 106 pfu per ml.

B. campestris cDNA Library Screen: The library is

plated on E. coli strain C600 hfl- at a density of
approximately 30,000 pfu/150mm NZY plate to provide
approximately 120,000 plaques for screening. Phage are
lifted in duplicate on to NEN (Boston, MA.) Colony/Plaque
Screen filters as described in Example 5. Filters are

prehybridized and hybridized with the 32p-labeled fragment
of pCGN3230 as described above for the Northern
hybridization. Filters are washed for 30 minutes in 2X
SSC, 0.1% SDS at 50°C and at 55°C twice for 15 minutes
each. Filters are exposed to X-ray film overnight at -80°C
with a Dupont Cronex intensifying screen.

Clones are detected by hybridization on duplicate filters to the R. communis desaturase cDNA fragment and plaque purified. During plaque purification, the probe used was a gel-purified 1.4 kb SstI fragment of pCGN3230 which lacks the poly(A) + tail. As described above, phage DNA is isolated from purified lambda clones, digested with EcoRI, ligated, and transformed to E. coli DH5\alpha cells. Minipreparation DNA is prepared and partial DNA sequence determined as described above. Partial DNA sequences of two clones, pCGN3235 and pCGN3236, are presented in Figure 4A (SEQ ID NO: 17) and 4B (SEQ ID NO: 18), respectively. Initial DNA sequence analysis of the 3' regions of these clones indicates that pCGN3236 and pCGN3235 are cDNA

clones from the same gene. pCGN3236 is a shorter clone than pCGN3235, which appears to contain the entire coding region of the *B. campestris* desaturase gene. The complete nucleotide sequence of pCGN3235 is presented in Figure 4C and SEQ ID NO: 19.

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Desaturase Gene Analysis: Southern and Northern analyses of Brassica species are conducted to determine the number of genes which encode the Brassica desaturase clone, pCGN3235 in B. campestris, B. oleracea, and B. napus, and the timing of expression of the gene in B. campestris developing seeds. DNA is isolated from leaves of each of the above-named Brassica species by the method of Bernatzky and Tanksley (Theor. Appl. Genet. (1986) 72:314-321). from each of the species is digested with restriction endonucleases EcoRI and XbaI (10 ug/digest), electrophoresed in a 0.7% agarose gel, and transferred to a nitrocellulose filter (Maniatis et al., supra). The filter is prehybridized and hybridized at 42°C (as described above for Northern analysis using R. communis desaturase clone) with a 32P-labeled (nick translation) gel-isolated HindIII/PvuII fragment of pCGN3235 (Fig. 7C). The filter is washed following overnight hybridization, for 30 minutes at 55°C in 1X SSC, 0.1% SDS, followed by two 15 minute

The autoradiograph indicates that the *Brassica* desaturase is encoded by a small gene family consisting of about two genes in *B. campestris* and *B. oleracea*, and about four genes in *B. napus*.

washed at 55°C in 0.1% SSC, 0.1% SDS.

The timing of expression of the desaturase gene during seed development is determined by Northern analysis. RNA is isolated from immature seeds of B. campestris cv. R500 collected at 11, 13, 15, 17, 19, 21, 25, 30, 35, and 40 days post-anthesis. Total RNA is isolated as described by Scherer and Knauf (Plant Mol. Biol. (1987) 9:127-134). Twenty five micrograms of RNA from each time point are electrophoresed through a formaldehyde-containing 1.5% agarose gel as described by Shewmaker, et al. (supra) and blotted to nitrocellulose (Thomas, Proc. Nat. Acad. Sci.

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(1980) 77:5201-5205). The blot is pre-hybridized and hybridized at 42°C with the <sup>32</sup>p-labeled HindIII/PvuII fragment of pCGN3235 as described above. The filter is washed following overnight hybridization, for 30 minutes at 55°C in 1X SSC, 0.1% SDS, followed by two 15 minute washed at 55°C in 0.1X SSC, 0.1% SDS.

The autoradiograph indicates that the desaturase gene is expressed in *B. campestris* developing seeds beginning at about day 19 and through about day 30, with maximal expression at day 25. By a similar Northern analysis, the level of desaturase mRNA in developing *Brassica napus* seeds (day 21) was estimated to be approximately 1% of the total mRNA.

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Isolation of Other Desaturase Gene Sequences: cDNA

libraries may be constructed as described above and genomic libraries can be constructed from DNA from various sources using commercially available vectors and published DNA isolation, fractionation, and cloning procedures. For example, a B. campestris genomic library can be constructed using DNA isolated according to Scofield and Crouch (J.Biol.Chem. (1987) 262:12202-12208) that is digested with BamHI and fractionated on sucrose gradients (Maniatis et al., supra), and cloned into the lambda phage vector LambdaGem-11 (Promega; Madison, WI) using cloning procedures of Maniatis et al. (supra).

cDNA and genomic libraries can be screened for desaturase cDNA and genomic clones, respectively, using published hybridization techniques. Screening techniques are described above for screening libraries with DNA fragments. Libraries may also be screened with synthetic oligonucleotides, for example using methods described by Berent et al. (BioTechniques (1985) 3:208-220). Probes for the library screening can be prepared by PCR, or from the sequences of the desaturase clones provided herein. Oligonucleotides prepared from the desaturase sequences may be used, as well as longer DNA fragments, up to the entire desaturase clone.

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For example, jojoba polyadenylated RNA is used to construct a cDNA library in the cloning vector λZAPII/EcoRI (Stratagene, San Diego, CA). RNA is isolated from jojoba embryos collected at 80-90 days post-anthesis by isolating polyribosomes using a method initially described by Jackson and Larkins (Plant Physiol. (1976) 57:5-10) and modified by Goldberg et al. (Developmental Biol. (1981) 83:201-217). Polysaccharide contaminants in the polyribosomal RNA preparation are removed by running the RNA over a cellulose column (Sigma-cell 50) in high salt buffer (0.5M NaCl, 20mM Tris pH 7.5, 1mM EDTA, 0.1% SDS). The contaminant binds to the column and the RNA is collected in the eluant. eluant fractions are pooled and the RNA is ethanol precipitated. The precipitated total RNA is then resuspended in a smaller volume and applied to an oligo d(T) cellulose column to isolate the polyadenylated RNA.

The library is constructed using protocols, DNA and bacterial strains as supplied by the manufacturer. Clones are packaged using Gigapack Gold packaging extracts (Stratagene), also according to manufacturer's recommendations. The cDNA library constructed in this manner contins approximately 1 x 10<sup>6</sup> clones with an average cDNA insert size of approximately 400 base pairs.

The jojoba library is plated on *E. coli* XL1-Blue (Stratagene) at a density of approximately 5000pfu/150mm plate to provide approximately 60,000 plaques for screening. Phage are lifted onto duplicate nylon membrane filters as described previously. Filters are prehybridized at 42°C in a hybridization buffer containing 40% formamide, 10X Denhardt's solution, 5X SSC, 0.1% SDS, 50mM EDTA, and 100µg/ml denatured salmon sperm DNA. Hybridization is at 42°C in the same buffer with added nick translated (BRL Nick Translation System) 520 bp *BglII* fragment of the *C. tinctorius* desaturase clone described previously. Filters are washed at 50°C in 2X SSC and exposed to X-ray film overnight.

Desaturase clones are detected by hybridization on duplicate filters with the *C. tinctorius* cDNA fragment and

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plaque-purified. Positive clones are recovered as plasmids in E. coli following manufacturer's directions and materials for in vivo excision. Partial, preliminary DNA sequence of a clone, 3-1, is determined and the corresponding amino acid sequence is translated in three frames. In this manner, homology to the C. tinctorius desaturase cDNA clone is detected in one reading frame. The preliminary DNA sequence of this jojoba desaturase cDNA fragment is shown in Figure 5 (SEQ ID NO: 43). Also shown is the corresponding translated amino acid sequence in the 10 reading frame having C. tinctorius desaturase homology. The jojoba cDNA fragment is approximately 75% homologous at the DNA level and approximately 79% homologous at the amino acid level compared to sequence of the C. tinctorius 15 desaturase in this region.

#### Example 13

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Antisense constructs are described which allow for transcription of a reverse copy of the *B. campestris* desaturase cDNA clone in the 5' to 3' orientation of transcription.

Preferential Expression of Antisense Constructs in Embryos In order to reduce the transcription of a desaturase gene in embryos of B. napus or B. campestris, constructs 25 may be prepared which allow for production of antisense copies of the desaturase cDNA preferentially in the embryos. Promoter sequences which are desirable to obtain this pattern of expression include, but are not limited to, the ACP, Bce4, and napin 1-2 expression cassettes described 30 in Examples 7, 8, and 9, respectively. It also may be desirable to control the expression of reverse copies of the desaturase cDNA under two different promoters in the same transformed plant to provide for a broader timing of expression of the antisense desaturase DNA. For example, 35 expression from the ACP promoter may begin and end earlier than expression from the napin promoter. Thus, expressing the reverse desaturase from both promoters may result in

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the production of the antisense strand of DNA over a longer period of embryo development.

An example of expression of an antisense desaturase gene preferentially in the embryos is provided below. Similar constructs containing the same or a different fragment of the desaturase gene and any of the promoters described above, as well as other promoter regions which may be useful, may also be prepared using gene cloning, insertion, mutation and repair techniques well known to those of ordinary skill in the art.

A. Antisense Desaturase Expression from the ACP Promoter Construction of pCGN3239 is as follows:

pCGN3235 (Example 12) is digested with PvuII and

HindIII and the HindIII sticky ends are filled in with

Klenow in the presence of 200 µM dNTPs. The 1.2 kb

PvuII/HindIII fragment containing the desaturase coding

sequence is gel purified and ligated in the antisense

orientation into EcoRV-digested pCGN1977 (ACP expression

cassette; described in Example 7) to create pCGN3238. The

4.2 kb XbaI/Asp718 fragment of pCGN3238 containing the

antisense desaturase in the ACP cassette is transferred

into XbaI/Asp718-digested pCGN1557 (binary transformation

vector; described in Example 7) to create pCGN3239.

25 B. Antisense Desaturase Expression From The Napin
Promoter

Construction of pCGN3240 is as follows: pCGN3235 is digested with PvuII and HindIII, the sticky ends are blunted, and the resulting fragment is inserted in an antisense orientation into pCGN3223 which has been digested with SalI and blunted with Klenow enzyme. The resulting plasmid, pCGN3240 will express an anti-sense desaturase RNA from the napin promoter cassette.

C. Antisense Desaturase Expression From a Dual Promoter Cassette

Construction of pCGN3242 is as follows: An Asp718 fragment of pCGN3240 containing the napin 5' and 3' regions surrounding the desaturase sequences is inserted into the

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Asp718 site of pCGN3239 (a binary vector containing an ACP promoter, antisense desaturase construct) to create pCGN3242.

- 5 Constitutive Transcription
  - A. Binary Vector Construction
  - 1. Construction of pCGP291.

The KpnI, BamHI, and XbaI sites of binary vector pCGN1559 (McBride and Summerfelt, Pl.Mol.Biol. (1990) 14:

269-276) are removed by Asp718/XbaI digestion followed by blunting the ends and recircularization to produce pCGP67. The 1.84 kb PstI/HindIII fragment of pCGN986 containing the 35S promoter-tml3' cassette is inserted into PstI/HindIII digested pCGP67 to produce pCGP291.

15 2. Construction of pCGN986.

The 35S promoter-tml3' expression cassette, pCGN986, contains a cauliflower mosaic virus 35S (CaMV35) promoter and a T-DNA tml 3'-region with multiple restriction sites between them. pCGN986 is derived from another cassette, 20 pCGN206, containing a CaMV35S promoter and a different 3' region, the CaMV region VI 3'-end. The CaMV 35S promoter is cloned as an AluI fragment (bp 7144-7734) (Gardner et. al., Nucl.Acids Res. (1981) 9:2871-2888) into the HincII site of M13mp7 (Messing, et. al., Nucl.Acids Res. (1981) 9:309-321) to create C614. An EcoRI digest of C614 25 produced the EcoRI fragment from C614 containing the 35S promoter which is cloned into the EcoRI site of pUC8 (Vieira and Messing, Gene (1982) 19:259) to produce pCGN147.

pCGN148a containing a promoter region, selectable marker (KAN with 2 ATG's) and 3' region, is prepared by digesting pCGN528 with BglII and inserting the BamHI-BglII promoter fragment from pCGN147. This fragment is cloned into the BglII site of pCGN528 so that the BglII site is proximal to the kanamycin gene of pCGN528.

The shuttle vector used for this construct, pCGN528, is made as follows: pCGN525 is made by digesting a plasmid containing Tn5 which harbors a kanamycin gene (Jorgenson

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et. al., Mol. Gen. Genet. (1979) 177:65) with HindIII-BamHI and inserting the HindIII-BamHI fragment containing the kanamycin gene into the HindIII-BamHI sites in the tetracycline gene of pACYC184 (Chang and Cohen, J. Bacteriol. (1978) 134:1141-1156). pCGN526 was made by inserting the BamHI fragment 19 of pTiA6 (Thomashow et. al., Cell (1980) 19:729-739), modified with XhoI linkers inserted into the SmaI site, into the BamHI site of pCGN525. pCGN528 is obtained by deleting the small XhoI fragment from pCGN526 by digesting with XhoI and religating.

pCGN149a is made by cloning the BamHI-kanamycin gene fragment from pMB9KanXXI into the BamHI site of pCGN148a. pMB9KanXXI is a pUC4K variant (Vieira and Messing, Gene (1982) 19:259-268) which has the XhoI site missing, but contains a functional kanamycin gene from Tn903 to allow for efficient selection in Agrobacterium.

pCGN149a is digested with HindIII and BamHI and ligated to pUC8 digested with HindIII and BamHI to produce pCGN169. This removes the Tn903 kanamycin marker. pCGN565 and pCGN169 are both digested with HindIII and PstI and ligated to form pCGN203, a plasmid containing the CaMV 35S promoter and part of the 5'-end of the Tn5 kanamycin gene (up to the PstI site, Jorgenson et. al., (1979), supra). A 3'-regulatory region is added to pCGN203 from pCGN204, an EcoRI fragment of CaMV (bp 408-6105) containing the region VI 3' cloned into pUC18 (Yanisch-Perron, et al., Gene (1985) 33:103-119) by digestion with HindIII and PstI and ligation. The resulting cassette, pCGN206, is the basis for the construction of pCGN986.

The pTiA6 T-DNA tml 3'-sequences are subcloned from the Baml9 T-DNA fragment (Thomashow et al., (1980) supra) as a BamHI-EcoRI fragment (nucleotides 9062 to 12,823, numbering as in Barker et al., Plant Mol. Biol. (1982) 2:335-350) and combined with the pACYC184 (Chang and Cohen (1978), supra) origin of replication as an EcoRI-HindIII fragment and a gentamycin resistance marker (from plasmid

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pLB41), obtained from D. Figurski) as a BamHI-HindIII fragment to produce pCGN417.

The unique SmaI site of pCGN417 (nucleotide 11,207 of the Bam19 fragment) is changed to a SacI site using linkers and the BamHI-SacI fragment is subcloned into pCGN565 to give pCGN971. The BamHI site of pCGN971 is changed to an EcoRI site using linkers. The resulting EcoRI-SacI fragment containing the tml 3' regulatory sequences is joined to pCGN206 by digestion with EcoRI and SacI to give pCGN975. The small part of the Tn5 kanamycin resistance gene is deleted from the 3'-end of the CaMV 35S promoter by digestion with SalI and BglII, blunting the ends and ligation with SalI linkers. The final expression cassette pCGN986 contains the CaMV 35S promoter followed by two SalI sites, an XbaI site, BamHI, SmaI, KpnI and the tml 3' region (nucleotides 11207-9023 of the T-DNA).

#### B. <u>Insertion of Desaturase Sequence</u>

The 1.6 kb XbaI fragment from pCGN3235 containing the desaturase cDNA is inserted in the antisense orientation into the XbaI site of pCGP291 to produce pCGN3234.

#### Plant Transformation

The binary vectors containing the expression cassette and the desaturase gene are transformed into Agrobacterium tumefaciens strain EHA101 (Hood, et al., J. Bacteriol. (1986) 168:1291-1301) as per the method of Holsters, et al., Mol. Gen. Genet. (1978) 163:181-187. Transformed B. napus and/or Brassica campestris plants are obtained as described in Example 10.

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### Analysis of Transgenic Plants

# A. Analysis of pCGN3242 Transformed Brassica campestris cv. Tobin Plants

Due to the self-incompatibility of Brassica campestris

cv. Tobin, individual transgenic plants are pollinated using non-transformed Tobin pollen. Because of this, the T2 seeds of a transgenic plant containing the antisense desaturase at one locus would be expected to segregate in a

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1:1 ratio of transformed to non-transformed seed. composition of ten individual seeds collected at 26 days post-anthesis from several pCGN3242-transformed plants and one non-transformed control was analyzed by gas chromatography according to the method of Browse, et al., Anal. Biochem. (1986) 152:141-145. One transformant, 3242-T-1, exhibits an oil composition that differed distinctly from controls on preliminary analysis. The control Tobin seeds contained an average of 1.8% 18:0 (range 1.5% - 2.0%) 10 and 52.9% 18:1 (range 48.2% - 57.1%). T2 seeds of 3242-T-1 segregated into two distinct classes. Five seeds contained levels of 18:0 ranging from 1.3% to 1.9% and levels of 18:1 ranging from 42.2% to 58.3%. The other five seeds contained from 22.9% to 26.3% 18:0 and from 19.9% to 26.1% 15 18:1.

## B. Analysis of pCGN3234 Transformed Plants

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Some abnormalities have been observed in some transgenic Brassica napus cv. Delta and Bingo and Brassica campestris cv. Tobin plants containing pCGN3234. These effects could be due to the constitutive expression of antisense desaturase RNA from the 35S promoter or could be due to the transformation/tissue culture regime the plants have been subjected to.

The above results demonstrate the ability to obtain plant \$\Delta - 9\$ desaturases, isolate DNA sequences which encode desaturase activity and manipulate them. In this way, the production of transcription cassettes, including expression cassettes can be produced which allow for production, including specially differentiated cell production of the desired product. A purified \$C\$, tinctorius desaturase is provided and used to obtain nucleic acid sequences of \$C\$, tinctorius desaturase. Other plant desaturase sequences are provided such as \$R\$, cummunis, \$B\$, campestris, and \$S\$, chinensis. These sequences as well as desaturase sequences obtained from them may be used to obtain additional desaturease, and so on. And, as described in the application modification of oil composition may be achieved.

by reference.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claim.

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## What is claimed is:

- A recombinant DNA construct comprising a sequence encoding at least a portion of a plant desaturase, said desaturase when mature having activity toward an unsaturated fatty acid substrate.
  - 2. The construct of Claim 1 encoding a biologically active plant desaturase.
  - 3. The construct of Claim 1 wherein said sequence encodes a precursor desaturase.
- 4. The construct of Claim 1 wherein said sequence encodes a mature desaturase.
  - 5. The construct of Claim 1 wherein said sequence encodes a transit peptide.
- The construct of Claim 1 comprising a cDNA
   sequence.
  - 7. The construct of Claim 1 wherein said sequence is joined to a second nucleic acid sequence which is not naturally joined to said first sequence.
- 8. The construct of Claim 1 comprising, in the 5' to
   3' direction of transcription, a transcriptional regulatory region functional in a host cell and said sequence.
  - 9. The construct of Claim 8 further comprising, a translational regulatory region immediately 5' to said sequence and a transcriptional/translational termination regulatory region 3' to said sequence, wherein said regulatory regions are functional in said host cell.
  - 10. The construct of Claim 8 wherein said sequence is a sense sequence.
- 11. The construct of Claim 8 wherein said sequence is 30 an anti-sense sequence.
  - 12. The construct of Claim 8 wherein said host cell is a plant cell.
  - 13. The construct of Claim 12 wherein said transcriptional initiation region is obtained from a gene preferentially expressed in plant seed tissue during lipid accumulation.
    - ,14. The construct of Claim 13 wherein said transcriptional initiation region is selected from the

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regulatory region 5' upstream to a structural gene of the group consisting of any one of Bce4, seed ACP Bcg 4-4 and napin 1-2.

- 15. The construct of Claim 9 wherein said transcriptional termination region is a plant desaturase termination region.
  - 16. The construct of Claim 1 wherein said plant desaturase is a  $\Delta$ -9 desaturase.
- 17. The construct of Claim 1 wherein said sequence is obtainable from any one of *C. tinctorius*, *R. communinis* and *B. campestris*.

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18. A method of modifying fatty acid composition in a plant host cell from a given percentage of fatty acid saturation to a different percentage of fatty acid saturation comprising

growing a host plant cell having integrated into its genome a recombinant DNA sequence encoding a plant desaturase under the control of regulatory elements functional in said plant cell during lipid accumulation under conditions which will promote the activity of said regulatory elements.

- 19. The method of Claim 18 wherein the overexpression of plant desaturase is obtained.
- 20. The method of Claim 18 wherein the decrease of 25 endogenous plant desaturase is obtained.
  - 21. The method of Claim 18 wherein said regulatory elements function preferentially in plant seed.
  - 22. The method of Claim 20 wherein the percentage of long chain unsaturated fatty acids is increased.
- 23. A plant cell having a modified level of saturated fatty acids produced according to the method of any one of Claims 18-22.
  - 24. The plant cell of Claim 23 wherein said cell is a Brassica plant cell.
- 35 25. The plant cell of Claim 23 wherein said cell is in vivo.
  - 26. The plant cell of Claim 23 wherein said cell is an oilseed embryo plant cell.

- 27. A plant seed having a modified level of saturated fatty acids as compared to a seed of said plant having a native level of saturated fatty acids produced according to a method comprising
- growing a plant, having integrated into the genome of embryo cells a recombinant DNA sequence encoding a plant desaturase under the control of regulatory elements functional in seed during lipid accumulation, to produce seed under conditions which will promote the activity of said regulatory elements, and

harvesting said seed.

- 28. The seed of Claim 27 wherein said plant is Brassica napus.
- 29. The seed of claim 27 wherein said seed is an oilseed.
  - 30. The seed of Claim 27 wherein said plant desaturase is a  $\Delta$ -9 desaturase.
  - 31. A plant seed oil of a plant having an endogenous level of saturated fatty acids comprising a plant seed oil having a modified level of saturated fatty acids.
  - 32. The oil of Claim 31 comprising a Brassica napus oil.
  - 33. A plant seed oil separated from an seed produced according to any one of Claims 27-30.
- 25 34. A host cell comprising a plant desaturase encoding sequence of any one of Claims 1-17.
  - 35. The cell of Claim 34 wherein said cell is a plant cell.
- 36. The cell of Claim 35 wherein said plant cell is 30 in vivo.
  - 37. The cell of Claim 35 wherein said plant cell is a Brassica plant cell.
  - 38. A transgenic host cell comprising an expressed plant desaturase.
- 35 39. The cell of Claim 38 wherein said host cell is a plant cell.
  - 40. The cell of Claim 38 wherein said plant desaturase is a  $\Delta$ -9 desaturase.

41. A method of producing a plant desaturase in a host cell or progeny thereof comprising

growing a host cell or progeny thereof comprising a construct of any one of Claims 1-10 and 12-17 under conditions which will permit the production of said plant desaturase.

- 42. The method of Claim 41 wherein said host cell is a plant cell and said construct is integrated into the genome of said plant cell.
- 10 43. The method of Claim 42 wherein said plant cell is in vivo.
  - 44. A host cell comprising a plant desaturase produced according to Claim 41.
- 45. The cell of Claim 45 wherein said host cell is a plant host cell and said construct is integrated into the genome of said plant cell.

 $\texttt{ASTLGSSTPKVDNAKKPFQPPREVHVQVTH}_{\textbf{X}}^{\textbf{S}} \texttt{MPPQKIEIFKSIEG}_{\textbf{R}}^{\textbf{W}} \texttt{AEQNILV}_{\textbf{F}}^{\textbf{L}} \texttt{KPVEKCWQ}$ DFLPDPA<sub>T</sub>EGFDEOVKELRARAKEIPDDYFVVLVGDMITEEALPTYOTMINTIDGV

되:

F3: DETGASLTPWAVWT

F2:

F4: DLLHTYLYLSGRV

F5: DMRQIQKTIQYLI

F6: TENSPYLGFIYTSFQER

 $\mathtt{DV}_{\overline{F}}^{K}\mathtt{LAQI}_{\overline{O}}^{C}\mathtt{GTIASDEKRHETAYTKIVEKLFEIDPDGTVLAFADMMRKKI}_{\overline{\mathbf{p}}}\mathtt{MPAHLMY}$ F7:

F8: DNLF

F9: dvFlAV<sub>I</sub>QRL<sub>I</sub>VYTAK

F10: DYADILEFLVGRWK

vadliglsgegrka<sub>g</sub>dyvcglpprirrleeraograkegpvvpfswifdrovkl F11:

FIGURE 1

69	138	207	276	345
HindIII     GCTCACTTGTGTGGAGAAAAAAAAACTCACAAAAAAGCTTTGCGACTGCCAAGAACAACAACA   42	70 ACAACAAGATCAAGAAGAAGAAGAAGATCAAAAATGGCTCTTCGAATCACTCCAGTGACCTTGCAA METAlaLeuArgIleThrProValThrLeuGln	ECORV	HindII       SerThrLeuGlySerSerThrProLysValAspAsnAlaLysLysProPheGlnProProArgGluVal   238	277 CATGTTCAGGTGACGCACTCCATGCCACCACAGAAGATAGAGATTTTCAAATCCATCGAGGGTTGGGCT HisValGlnValThrHisSerMETProProGlnLysIleGluIlePheLysSerIleGluGlyTrpAla

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FIGURE 2 Page 1 of 4

346 GAGCAGAACATATTGGTTCACCTAAAGCCAGTGGAGAAATGTTGGCAAGCACAGGATTTCTTGCCGGAC GluGlnAsnIleLeuValHisLeuLysProValGluLysCysTrpGlnAlaGlnAspPheLeuProAsp

FIGURE 2 Page 2 of 4

829 CATGCAAAGGATCATGGGGACGTGAAACTGGCGCAAATTTGTGGTACAATCGCGTCTGACGAAAAGCGT

SphI

HisAlaLysAspHisGlyAspValLysLeuAlaGlnIleCysGlyThrIleAlaSerAspGluLysArg

ClaI

HisGluThrAlaTyrThrLysIleValGluLysLeuPheGluIleAspProAspGlyThrValLeuAla 898

996

BglII

TITGCCGACATGATGAGGAAAAAGATCTCGATGCCCGCACCTTGATGTACGATGGGCGTGATGACAAC 1035 PheAlaAspMETMETArgLysLysIleSerMETProAlaHisLeuMETTyrAspGlyArgAspAspAsn 196

AccI

1036 CTCTTCGAACATTTCTCGGCGGTTGCCCAAAGACTCGGCGTCTACACGCCCAAAGACTACGCCGACATA 1104 LeuPheGluHisPheSerAlaValAlaGlnArgLeuGlyValTyrThrAlaLysAspTyrAlaAspIle

1105 CTGGAATTTCTGGTCGGCGGTGGAAAGTGGCGGATTTGACCGGCCTATCTGGTGAAGGGGCGTAAAGCG  ${ t LeuGluPheLeuValGlyArgTrpLysValAlaAspLeuThrGlyLeuSerGlyGluGlyArgLysAla}$ 

FIGURE 2 Page 3 of Saci

1174 CAAGATTATGTTTGCGGGTTGCCACCAAGAATCAGAAGGCTGGAGGAGAGGTCAAGGGCGAGCAAAG 1242 GlnAspTyrValCysGlyLeuProProArgIleArgArgLeuGluGluArgAlaGlnGlyArgAlaLys

PvulI

GluGlyProValValProPheSerTrpIlePheAspArgGlnValLysLeu 1312 GCAGTGAGTTCGGTTTCTGTTGGCTTATTGGGTAGAGGTTAAAACCTATTTTAGATGTCTGTTTCGTGT 1380

1381 AAIGTGGTTTTTTTTTTTTTTAATCTTGAATCTGGTATTGTGTCGTTGAGTTCGCGTGTGTAAACTTG 1449

1450 TGTGGCTGTGGACATATTATAGAACTCGTTATGCCAATTTTGATGACGGTGGTTATCGTCTCCCCTGGT 1518

1519 GTTTTTTTTTT 1533

Page 4 of FIGURE 2

69 1 AAAAGAAAAAGGTAAGAAAAAAAAAAGGCTCTCAAGCTCAATCCTTTCCTTTCTCAAACCCAAAAGT METAlaLeuLysLeuAsnProPheLeuSerGlnThrGlnLysL

BgllI

70 TACCTICITICGCICTICCACCAATGGCCAGIACCAGAICICCIAAGTICTACATGGCCICTACCTCA 138 euProSerPheAlaLeuProProMETAlaSerThrArgSerProLysPheTyrMETAlaSerThrLeuL

139 AGTCTGGTTCTAAGGAAGTTGAGAATCTCAAGAAGCCTTTCATGCCTCCTCGGGAGGTACATGTTCAGG ysSerGlySerLysGluValGluAsnLeuLysLysProPheMETProProArgGluValHisValGlnV

208 ITACCCATTCTATTGCCA 225 alThrHisSerlleAla

FIGURE 3A

<b>VO 91/1</b> :	3972				,	7/48		PCT/	US91/01746
56	110	164	218	272	326	380	434	488	٠.
EH	TCT	AAT Asn	TCT	GAG Glu	TTT Phe	GAG Glu	ATA	GTT Val	-
r rcr u Ser	aga Arg	GAG Glu	CAT His	GAG Glu	GAT Asp	AGG Arg	ATG MET	GGA Gly	
C CTT	ACC Thr	GTT Val	ACC Thr	GCT	CAG Gln	CIC	gac Asp	GAT Asp	
rTC Phe	AGT	GAA	GTT Val	TGG	CCG	GAA Glu	GGA Gly	rrg Leu	
CCT Pro	GCC	AAG Lys	CAG Gln	AAT Asn	CAA	AGG Arg	GTT Val	ACC Thr	
AAT Asn	ATG	TCT	GTT Val	GAC	TGG	GTC Val	TIG	AAT Asn	
CIC	CCA	GGT Gly	CAT His	CTA	TGT Cys	CAA	GTT Val	CIG	
AAG Lys	CCA	TCT	GTA	TCC	AAA Lys	GAG Glu	GTT Val	atg Met	3B £ 3
r CTC	CTT Leu	AAG Lys	GAG Glu	AAA Lys	GAG Glu	GAT Asp	TTT Phe	ACA Thr	0
GCT PALA	GCT	CTC	CGG Arg	TTT Phe	GTT Val	TTT Phe	TAT Tyr	CAA	FIGURE
ATG MET	TTC	ACC	CCT	ATC Ile	CCA	GGA Gly	GAT Asp	TAT	Δι
AAAACA	TCT	TCT	CCT	GAG Glu	AAG Lys	gat Asp	GAT Asp	ACT	
	CCT	GCC	ATG	ATT Ile	CTG	TCT	CCT	CCC	•
GAAA	TTA (	ATG	TTC Phe	AAG Lys	CAT His	GCC	ATT Ile	CIT	
GTAA	AAG Lys	TAC	CCT Pro	CAA Gln	GTT Val	CCC	GAG	GCC	
AA G	CAA Gln	TTC	AAG Lys	CCC Pro	CTG	GAT	AAG	GAA Glu	
GAAA	ACC	AAG	AAG	CCA Pro	ATT (Ile	CCA (Pro /	GCA A	GAA (	
AAAAGAAAA GGTAAGAAAA	CAA G	CCT 2 Pro ]	CTC 7	ATG (	AAC A	TTG (	AGA (Arg A	ACG OTHE	

)	91/1397	2				8/	42		PCT/		
	542	596	650	704	758	812	866	920	974		
	TGG	TCT	TCA	TCA	aaa Lys	GAG Glu	GAT Asp	ATG MET	TCA Ser		
	GCA Ala	CTA	GGT	ACA Thr	GCC	GAT Asp	ATT Ile	TCT	TTT Phe		
	AGG Arg	TAC	ATT Ile	TAT Tyr	CAA Gln	GCA	GAG Glu	ATT Ile	CAC		
	ACA Thr	CTC	TTG	ATC Ile	CGA	GCT	TTT Phe	AAA Lys	GAC		
	TGG	TAT Tyr	ТАТ Туг	TTC	GCC	ATT	CTC	AAG Lys	TTT		
	ATT	aag Lys	CAA Gln	666 61y	ACT	ACA	aaa Lys	AGA Arg	CTT		
	GCA Ala	AAT Asn	ATT	CTT	AAC	GGT	GAA Glu	ATG	AAT		
	TGG Trp	CIC	ACA Thr	TAC	666 61y	TGT	GTG	ATG	GAT		
	TCT	CIC	AAG Lys	CCA	CAT His	ATA Ile	ATA	GAT	GAT Asp		
	ACT Thr	GAC Asp	GAG Glu	AGT	TCT	CAA	AAG	GCT	CGA Arg		
	CCT	GGT Gly	ATT Ile	AAC	ATT Ile	GCT	ACA	TTT Phe	GGC (		
	AGT Ser	CAT	CAA Gln	GAA Glu	TTC	TTG	TAC	GCT A	GAT ( Asp (		
	GCA Ala	AGA	AGG Arg	ACA	ACC	AAG Lys	GCC	TTG (	TAT (Tyr )		
	GGT	AAT Asn	ATG	CGG	GCA	ATA Ile	ACA	GTT	ATG '		
	ACA	GAG	GAC	CCA	AGG	GAC	GAG Glu	ACT (Thr	TTG 1		
	GAA	GAA Glu	GTG	GAT Asp	GAA Glu	GGA GIY	CAT (His (	GGA A	CAC 7 His 1		
	GAT	GCG	CGA Arg	ATG MET	CAG (Glu (	CAT (His (	CGC (Arg P	GAT (Asp (	GCA C		
	CGG (Arg )	ACT (Thr 1	GGA (Gly A	GGA A	TTC (Phe (	GAG (Glu F	AAG CLYS P	CCT G Pro A	CCT G Pro A		
	) i				Η	ن ب	R, H	Од	D M		

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FIGURE 3B Page 2 of 3

O 91/13972				91	142				PCT	/US91/01746
1028	1136	1190	1254	1324	1394	1464	1534	1604	1668	
GCT GTT GCG CAG CGT CTT GGA GTC TAC ACA GCA AAG GAT TAT GCA GAT ATA TTG Ala Val Ala Gln Arg Leu Gly Val Tyr Thr Ala Lys Asp Tyr Ala Asp Ile Leu GAG TTC TTG GTG GGC AGA TGG AAG GTG GAT AAA CTA ACG GGC CTT TCA GCT GAG Glu Phe Leu Val Gly Arg Trp Lys Val Asp Lys Leu Thr Gly Leu Ser Ala Glu	GGA CAA AAG GCT CAG GAC TAT GTT TGT CGG TTA CCT CCA AGA ATT AGA AGG CTG Gly Gln Lys Ala Gln Asp Tyr Val Cys Arg Leu Pro Pro Arg Ile Arg Arg Leu	GAA GAG AGA GCT CAA GGA AGG GCA AAG GAA GCA CCC ACC ATG CCT TTC AGC TGG Glu Glu Arg Ala Gln Gly Arg Ala Lys Glu Ala Pro Thr MET Pro Phe Ser Trp	ATT TTC GAT AGG CAA GTG AAG CTG TAGGTGGCTA AAGTGCAGGA CGAAACCGAA ATGGTTAGTT 1 Ile Phe Asp Arg Gln Val Lys Leu	TCACTCTTTT TCATGCCCAT CCCTGCAGAA TCAGAAGTAG AGGTAGAATT TTGTAGTTGC TTTTTTATTA 1	CAAGTCCAGT TTAGTTTAAG GTCTGTGGAA GGGAGTTAGT TGAGGAGTGA ATTTAGTAAG TTGTAGATAC 1	AGTTGTTTCT TGTGTTGTCA TGAGTATGCT GATAGAGAGC AGCTGTAGTT TTGTTGTTGT GTTCTTTAT 1	AIGGICICIT GIAIGAGITI CITITCITIC CITITCITCI TICCITICCI CICICICI	CTCTTTTTCT CTTATCCCAA GTGTCTCAAG TATAATAAGC AAACGATCCA TGTGGCAATT TTGATGATGG 1	TGATCAGTCT CACAACTTGA TCTTTTGTCT TCTATTGGAA ACACAGCCTG CTTGTTTGAA AAAA	FIGURE 3B Page 3 of 3

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FIGURE 4A

69

HindIII

70 ATGGCATTGAAGCTTAACCCTTTGGCATCTCAGCCTTACAACTTCCCT 117 METAlaLeuLysLeuAsnProLeuAlaSerGlnProTyrAsnPhePro

11/42

PstI

69 1 ACTICATGGGCTATTTGGACAAGAGCTTGGACTGCAGAAGAGAACCGACACGGTGATCTTCTCAATAAG ThrSerTrpAlaIleTrpThrArgAlaTrpThrAlaGluGluAsnArgHisGlyAspLeuLeuAsnLys 70 TATCTTTACTTGTCTGGACGTGTTGACATGAGGCAGATTGAAAAGACCATTCAGTACTTGATTGGTTCT 138 TyrLeuTyrLeuSerGlyArgValAspMETArgGlnIleGluLysThrIleGlnTyrLeuIleGlySer

BamHI

139 GGAATGGATCCTAGAACAGAGAACAATCCTTACCTCGG 176 GlyMETASpProArgThrGluAsnAsnProTyrLeuAla FIGURE 4B

pcGN3236

pcGN3235

TGAGAGATAG TGTGAGAGCA TTAGCCTTAG AGAGAGAGA AGAGAĠCTTG TGTCTGAAAG AATCCACAA

TCG TCC Pro CCT TIC Asn AAC Tyr TAC Pro CCI CAG Gln Ser TCI GCA Ala TIG Leu Pro CCT Asn AAC Len CTT AAG Lys Leu TIG Ala GCA ATG

TCI GCT CIC Len TGC Cys CIC Leu TTC AAG ည္သ Pro TCT Ser AGA Arg TIC Phe ACT  $\mathtt{TCT}$ Ser ATC Ile CCA Pro . (CCC) Pro CGI Arg GCT Ala

ACA TIC Pro CCA Lys AAG Lys AAG TTG Leu Ser AGT GAG Glu GIT Val Glu GAG AAG  $\mathbf{I}^{\mathsf{CC}}$ Ser AGC Ser CIC Leu Ala GCT ည္သ TCT TCL

ATC Ile AAG Lys CAG Gln CCC CCA Pro ATG MET ICC Ser CAT His CIG GTC Lys CAA Gln GTT Val CAC His GTG Val GAA Pro AAG Lys Ser CCI Pro CCA Ser

CAG Gln Thr ACT Leu CTA Leu CTT Asn AAC Gln CAG GAG Glu GCC  $\mathbf{IGG}$ Trp GAC GAA Glu ATG MET JCC Ser AAA Lys TIC Phe ATC GAG Glu

CTC

TCC GCA CCT GAC CCC TTA Leu TTC Phe GAC CAG Gln CCC CAG Gln TGG Trp TCG Ser AAG Lys GAG Glu GTG Val GAC Asp AAA Lys

12/42

CCT CIC Leu Glu GAG AGA Arg GCA Ala AGG Arg GAG Glu AGA Arg CTA Glu GAG AGA Arg GTT Val CAG Gln Asp GAT GAA Glu TTC Phe 9999 GAT Asp

ပ္ပင္ပင္ပ Pro CTTLeu 909 Ala GAG Glu GAA Glu ACG ATC Ile ATG MET GAC GGA Gly GTG Val CTG GTT Val GTT Val TIC Phe Tyr TAC GAT Asp GAT

FIGURE 4C Page 1 of 3

FIGURE 4C Page 2 of 3

GCT GCT Arg AGG Arg ACA ACC AAG Lys ATG TAC GGC AAC Asn ATG MET GCC CIC ATG MET AGA Arg Leu ACA GIG Val Pro Asp GAG Glu GAC Asp CCI AGA Arg GAC GAG Glu ACT TTG ACT GAA Glu Asp GAA Glu GAA Glu GGA Gly GTT Val GAT CAT His CAC His GGT Gly GAT GCA CGT ATG MET CAA Gln CAC His Arg CGT Asp GCT GAT AGG Arg ACT GAG Glu AAG Lys GGA GGA Gly TTC CCT Gly Pro CCT GTG Val TGG TCT TCT Ser TCA AAA Lys GAG Glu GAT Asp ATG GGA Gly GCT TTG GCC TCG GGT G1yGAC ATT Ile ACT GAT Asp AGA CAA ATC Ile TAC Ile TAC GCA Ala GAG Glu ATT TTG ACA Ţhr Leu ATC Ile CII Leu TIG ၁၅၁ Arg GCT Phe AAA Lys TTT TGG ACT Trp TAT TAC TyrTTC GCT ATA CIC Leu AAG AAC Asn Ile AAG Lys Gln ATT CAG 660 61y ACA Thr ACA AAG Lys AGG Arg TIG Leu GCT AAT Asn ATT Ile Asn GAG Glu ATG CTC AAC 66C 61y ATG TGG CIC Thr Leu ACC TAC Tyr GGA Gly TGC Cys GTT Val ATG ACC TCA CTT AAG Lys His ATC Ile CCT Pro CAC ATA GAC CAA Gln GAT Asp Glu GAA ACT Ser CAA Gln AAT Asn TCT AAG Lys GCA AAC Asn TAT CCC Pro GGT Gly ATT Ile ATC Ile GCC ACC TTT Phe AGC GAG Glu ACC CAC CAG Gln TTC CTA TAC GCG

GAC

TTT

CIC

GAA

GAT

999

GAT Asp

GGT Gly

TGG

TAC

AGA Arg

14/42

TAA AAAGGAA CAAAGCTATG AAACCTTTTC ACTCTCCGTC GTCCCTCATT TGATCTATCT Len AGG GAG GCA GTGGlu Val AGG Arg 999 G1yCAA CAA Gln GAA Gln CAT GAC AGA (His Asp Arg ( CAG Gln SCG GIT Ala Val GCT Ala Ala TTC AAA Lys AGA Arg GCT TIT GTT Val Phe AAC Asn GAG Glu ATA (Ile I Ser GGA ICI GAG Glu G1yGAT Asp Trp Ser Glu TGG TCT CTTGAA TIG Leu Leu Phe AGC ATT Ile AGG Pro Phe Ser GGT 61yAsn AAC GAC Ser AGG TIC TCA Asp GCG Ala ATC Ile CCI Len CTT Phe TAT Gly Val 999 AGA Arg GIT CCC AAG (Pro Lys V Leu GAC Asp ACC Thr CCA Ser AGC AAA Lys Leu ACT TIG Glu GCC Ser Gly AGC TTG GGA Asp ACT Thr GAG Glu GGG G1y AAA Lys Arg TAC AAG Lys Ile TGT ATT GLyGIT Lys TIG Val AAG Leu ပ္သင္ဟ Ala CTC GCTCTTGAAA TTGGTGTAGA TTACTATGGT TTGTGATATT GTTCGTGGGT CTAGTTACAA AGTTGAGAAG GTAGCTITGI IGTITCCAGI CTITAAAIGT ITTIGIĞITI GGICCTITIA GIAAACTIGI GT TGTAGTTAAA TCAGTTGAAC TGTTTGGTCT CAGTGATTTA

CAG Gln

Page 3 of FIGURE

,	5	142
•	•	, , -

48	96	143
AAG Lys	GAG Glu	AA
CAA Gln 15	TTG	GAG Glu
GTG Val	TCC Ser 30	GTG Val
CAT His	AAA Lys	CCT Pro 45
GCT	TTC	AAA Lys
GAA	ATT Ile	CTT
AGA Arg 10	GAG Glu	CAT
CCT	ATT Ile 25	GTG Val
CCT	AAG Lys	TTG Leu 40
ATG MET	CAA Gln	GTC Val
CAC	CCT	AAT Asn
CCT Pro 5	CCG	GAG Glu
ANG Xaa	ATK Xaa 20	GAG Glu
AAA Lys	TCA	GCT Ala 35
GCC Ala	CAT His	TGG Trp
GAT Asp 1	ACC	GGT Gly

IGURE

H N I	CAA ACN AUG CUN AAU AC/N G					16/	42	•	GTQ TGN TAC GAN TTP TGCTTAAGCGA 5'				
ø	CAA								GTO				
FVVLVGDMITEEALPTY Q			Forward Primers:	GAP ATO CCA GAO GAO TA3' Desat 13-1 A CCG Desat 13-2	Desat 13-3	Desat 13-4		Reverse Primers: (complements)	Desat 13-5a 3' Desat 13-6a				
*	UAU C		FO	ra3'				9 O	ΔΩ				
۵	GAU C			SAQ 3				•					
۵	GAU GAU UAU			SAQ (									
Ωı	CCN			CGA C	ပ္ပ	į S							
н	GAA AUU CCN G C	Ø		ATO (	•	•							
ធ	GAA G			3AP 1		•							
×	AAA									Ses		٤	J.
Amino Acid Sequence From Fragment F2	•			5'GCTAAGCTT AAP						Oligonucleotides	P = A Or G		1 ) 'V   N

FIGURE 6



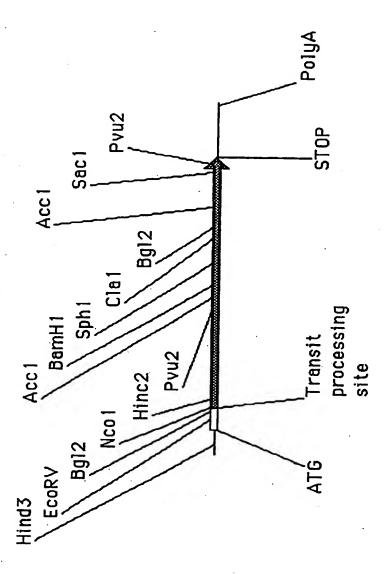


FIGURE 7A

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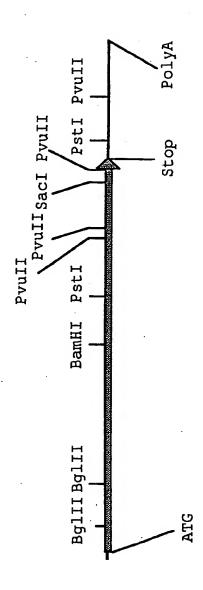


FIGURE 7B

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FIGURE 7C

980	AAAAAAAA	TCTTGTGCAC	ACAAATACTT CAATAAAAG ACCAAAAAA ATTAAAGCAA AGAAAAGCCT TCTTGTGCAC AAAAAAAAA	ATTAAAGCAA	ACCAAAAAA	CAATAAAAAG	ACAAATACTT
910	TCCCAATCTC	ATACCTCAAA	TATATATAAT ACCAGCATTC ACCATCATGA ATACCTCAAA TCCCAATCTC	ACCAGCATTC	TATATATAT	ATATAAATC TCCCCATCTC	ATATAAATC
840	TTACGACCAC	TATTTGTCCG	TGTAATGGCC ACTTGCAAGA GCGTTTCTTC AACGAGATAA GTCGAACAAA TATTTGTCCG TTACGACCAC	aacgagataa	GCGTTTCTTC	ACTTGCAAGA	rgtaatggcc
770	GATGGCCAAG	TCTTCCGCAT	CTGCACGAAA CTTGTGTGAG CATATTTTGT CTGTTTCTGG TTCATGACCT TCTTCCGCAT GATGGCCAAG	CTGTTTCTGG	CATATTTTGT	CTIGIGIGAG	CTGCACGAAA
7007	TGGTTTATTA AATCAACAAC	TGGTTTATTA	AATCAGAATT	CACAGCTGTT	ATTTATAAAA	GTCTGCTACA TCTGTCTTTT	STCTGCTACA
2069	TAAAAAATTA	CAAACTCTGG TAAAAAATTA	AAATACGTGT	AAATTGTCAT	AACTITIGIC AAAIIGICAI	TTTGTTGACT ACCGTATTGT	<b>LTTGTTGACT</b>
260	AATGGCAACG	TTTAGTTGGT	AATICITCAA ATCCITAAAA ATCAATCTCC CACTATTAAT CCCCCTTAGT ITTAGTIGGT AATGGCAACG	CACTATTAAT	ATCAATCTCC	ATCCTTAAAA	AATTCTTCAA
490	AGAATCTTCA	CAATAACAAT	CAA GACTTTTAA AAATTTAAGA ATAATATAAG CAATAACAAT AGAATCTTCA	AAATTTAAGA	GACTTTTAA	AATAACACAA	TCAACACACC AATAACA
420	ATTTATATGG AATTATAAAA	ATTTATATGG	GTTAGAAAAT TGTTGTACCA ATAACAAAG	TGTTGTACCA	GTTAGAAAAT	AATTAATTGA	TAATAAAAA AATTAAT
350	GCTTTTTAA	AAAATACTCA	TIGTGTAACA AGAATTAAAA AAAAAACAG AAAATACTCA GCTTTTTTAA	AGAATTAAAA		TTTTTGTGT AACAAATATT	ITTTTTGTGT
280	ATTGAAATTA TAAAGTGACA		TATAATTTGT	TGAAAATAAG	AATTTCCAGC	AATCAATGCA GTTTACAATT AATTTCCAGC TGAAAATAAG	AATCAATGCA
210	TTAGTTTTAA	CAGGGTCTCG	TTAAATAAAT AACCAAAAAC CAAAAATTCA TATCCGCGCGT GGCGCGGGT CAGGGTCTCG TTAGTTTTAA	TATCCGCGCT	CAAAAATTCA	AACCAAAAAC	<b>TAAATAAAT</b>
140	ATTAGAAATA CATCATTTAG	ATTAGAAATA	GTGAATATAA TACGTTAAAT		TATTTACTTG	GGTTTAAGAT GCCAAAAAT	GGTTTAAGAT
70	ATAAATATAT	AATATTCGTC	CTATTTTTA TATGATATCA AATATTCGTC ATAAATATAT	CTATTTTTA	TCIGITIGII	TCIAGAATIC ICTAATIACG ICTGITIGII	TCTAGAATTC

FIGURE 8 Page 1 of

					21/	42			
1036	1090	1144	1198	1252	1306	1369	1439	1509	1579
GAAGCCTTCT AGGTTTTCAC GAC ATG AAG TTC ACT ACT CTA ATG GTC ATC ACA TTG MET Lys Phe Thr Thr Leu MET Val Ile Thr Leu	GTG ATA ATC GCC ATC TCG TCT CCT GTT CCA ATT AGA GCA ACC ACG GTT GAA AGT Val Ile Ile Ala Ile Ser Ser Pro Val Pro Ile Arg Ala Thr Val Glu Ser	TTC GGA GAA GTG GCA CAA TCG TGT GTT GTG ACA GAA CTC GCC CCA TGC TTA CCA Phe Gly Glu Val Ala Gln Ser Cys Val Val Thr Glu Leu Ala Pro Cys Leu Pro	GCA ATG ACC ACG GCA GGA GAC CCG ACT ACA GAA TGC TGC GAC AAA CTG GTA GAG Ala MET Thr Thr Ala Gly Asp Pro Thr Thr Glu Cys Cys Asp Lys Leu Val Glu	CAG AAA CCA TGT CTT TGT GGT TAT ATT CGA AAC CCA GCC TAT AGT ATG TAT GTT Gln Lys Pro Cys Leu Cys Gly Tyr Ile Arg Asn Pro Ala Tyr Ser MET Tyr Val	ACT TCT CCA AAC GGT CGC AAA GTC TTA GAT TTT TGT AAG GTT CCT TTT CCT AGT Thr Ser Pro Asn Gly Arg Lys Val Leu Asp Phe Cys Lys Val Pro Phe Pro Ser	TGT TAAATCTCTC AAGACATTGC TAAGAAAAT ATTATTAAAA ATAAAAGAAT CAAACTAGAT Cys	CIGATGIAAC AAIGAATCAT CAIGTIAIGG ITGAAGCITA TAIGCIGAAG IGTIIGAITT TAIATAIGIG	TGTGTGTGTG TCCTGCTCAA TTTTTGAAAC ACACACGTTT CTCCTGATTT GGATTTAAAT TATATTTTGA	GTTAAAAAA AGAAAAAAGAT GGAATGCTAT TTATACAAGT TGATGAAAAA GTGGAAGTAC AATTTAGATA

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FIGURE 8 Page 2 of 4

PCT/US91/01746

ן /נע נ	3972						2/4	2				P	CT/I
1649	1719	1789	1859	1929	1999	2069	2/4 6812	2209	2279	2349	2419	2489	2559
ATTGTCGACA	CAGTICAACC AAATGATATT	TACCAGAAAC	rccercccc	CAATCAAAAA	TTCTCGTAAA	GGACAATTGT	AATGACATTC	ATAAAAACAA	TCAGATTCGA	TGAAACTGTT	CTAATTCCAA	GGCTTCTATC	CCCTGGCCG
TCTCCTACAC TTAAAGAATG AAACAATAAT AGACTTACGA AACAAATGAA AAATACATAA ATTGTCGACA	CAGTICAACC	GGGTGTCGTT	CTCTTTTTCT CAGCTCGCTA AAACCCTACC ACTAGAGACC TAGCTCTGAC CGTCGGCTCA TCGGTGCCGG	ATGACCCGAA ACCICTITI CCCAACTCAC GAAAACCCIA CAATCAAAAA	CATCGGTGCC GAAGGTGTAA CCTTTCTCTC CCATCATAGT	ATTTCTTATA	TTTTGTCTCA AAAGTGACAC TAGAAGAAAA AAGTCACAAA AATGACATTC	GGTTTAAATT AAATAAGTAA ACAAAAATAA ATAAAAACAA	TTATATGTTT	TGAAATTTTT	ATTTTATAAA ATTTTAAACG CTAATTCCAA	TITICICITI CITAIAITIG GGCIICIAIC	CAAAGTATCA TGTGTAACAA CCGGTGTTCA AAAACGCGCC CGCCTGGCCG
AACAAATGAA	GAAGGACTAG	ATGATAATCA TAAAAGAGAG AATGGGGGGG	TAGCTCTGAC	CCCAACTCAC	CCTTTCTCTC	GATTTTTTAA TGTTTAAACC ATGCCAAGCC	TAGAAGAAAA	AAATAAGTAA	GAAAAAAGA AATTTTTTTA TAGTTTCAGA	TTTTTTTTT	ATTTTATAAA	TTTTCTCTTT	CCGGTGTTCA
AGACTTACGA	TTATTATTAA AAATTTGTGT	Taaaagagag	ACTAGAGACC	ACCICICITI	GAAGGTGTAA	TGTTTAAACC	AAAGTGACAC		AATTTTTTA	TTAAATTTTC TTTTTTGAAA TTTTTTTTT	TTTATTTTT	CICCIAGICI	TGTGTAACAA
AAACAATAAT	TTATTATTAA	ATGATAATCA	AAACCCTACC		CATCGGTGCC	GATTTTTAA	TTTTGTCTCA	TAATACCTTT	GAAAAAAGA	TTAAATTTTC	TTTTTAGTA	CCCCAATICT	CAAAGTATCA
TTAAAGAATG	GATGACGAGT	GAACATATAC ATCAACAAT	CAGCICGCIA	AGGIGTAACC TITCTITCCC	CCTAGCTCCG ACCGTCGGCT	TGAAAGCTAA TTGGGCAATC	CAATAATAGC ATCTTTTGAG	ATTAAAAGT AAAATATCCC	AATAAAAAT	ATTCCCTTTT	TTATTTTAA	وزودودودو	TTTTCAGGCC
TCTCCTACAC	ATCAACGICC GAIGACGAG	GAACATATAC	CTCTTTTTCT	AGGTGTAACC	CCTAGCTCCG	TGAAAGCTAA	CAATAATAGC	ATTAAAAAGT	ATAAAATAAA AATAAAAAT	AATTTTTAA ATTCCCTTTT	TTTAAAATTT TTATTTTAA	AACTCCCCC CCCCCCCC	TTCTCTTTT TTTTCAGGCC

FIGURE 8 Page 3 of 4

TITACICGCC CGAITAAAIG AIGAICGGAA GGCIGCCAIG GCGAGGCGGA GGIAAICAGI GGIICIAGGC

ATATITAGIT GAAACICACA AGAATCGGIT GIAAAAACIA IGAAAICGIG CAAAAAAAI GAAGAACAAA

AAACTAGGCG

GCTGAAACTA GAAAACCTTC AAAAATCGAA ATTTTAAGAG CTAAATCGGT GTTTATCTCA

2699

TGAATCTATT

2769

Page, 4 of FIGURE

ပ

AATICGCCCT AIAGIGAGIC GIATIACGCG CGCICACIGG

69	<b>ω</b>	7	9		24/4		2		_	•	
	138	207	276	345	414	483	552	621	069	759	
1 CTCGAGAGCTGAAGGATTTTTTTTTAGAGATTCAACGACAGATGGACCCTTCCTCCACTAGGCAACTGC 2	70 AAGAACCTAACAATGCAAATATCACTCCTCCTCCAGCCTTCAAGGAGCGTTAATAGGACTGGAACAAGCG	B9111   139 GTCAAGTGAGTTTTTCCTTCCAAGATGATCTCTATGGTTCGGTTCATGAAGTTTGTGGTTTAATT 169	208 GIGIAGCAACAGGATAGIGCAAGIGAGAATAGAGTICGACCICAICIACCIACCCGGAACCICIGAAI	277 GTATCCCCATTGAAGAAGAAGAGGGCAAATCCTGCACCCAGAAGGATAAAGAAATTTTGGACGCCTGAA	346 GAAGTGGCAGTTCTGAGGGAAGGAGTAAAAGAGTATGTCTACTACTACTACTCTATAATCAAGTTTCAA	415 GAAGCTGAGCTTGGCTCTCACTTTATATGTTTGATGTTGTTGTGGGAGGTATGGTAAATCATGGAAAGAG	4 ATAAAGAATGCAAACCCTGAAGTATTGGCAGAGGACTGAGGTGAGAGAGCATGTCACTTTTGTGTTA	3 CTCATCTGAATTATCTTATATGCGAATTGTAAGTGGTACTAAAAGGTTTGTAACTTTTGGTAGGTGGAT	2 TTGAAGGATAAATGGAGGAACTTGCTTCGGTAGCGGTAACAAGTTTTATATTGCTATGAAGTTTTTTG	691 CCTGCGTGACGTATCAGCAGCTGTGGAAGATGGTATTAGAAAGGGTCTTTTCACATTTTGTGTTGTG	
	•	H	2(	2	34	41	484	553	622	69	

FIGURE 9 Page 1 of 6

760	760 ACAAATATTAATTCGGCCGGTATGGTTTGGTTAAGACTTGTTGAGAGACGTGTGGGGGTTTTTTGATGTA	828	
829	829 TAATTAGTCTGTGTTTAGAACGAAACAAGACTTGTTGCGTATGCTTTTTTTAACTTGAGGGGGTTTGTT	897	
868	Bglii     GTTGTTAGTTAGGAACTTGACTTTGTCTCTTCTCTCAAGATCTGATTGGTAAGGTCTGGGTGGTAGTA   937	996	
967	967 CTGTTTGGTTTAATTTGTTTTGACTATTGAGTCACTGTGGCCCATTGACTTTAAATTAGGCTGGTATAT 1035	1035	
1036	TITITGGTTTAAAACCGGTCTGAGATAGTGCAATTTCGATTCAGTCAATTTTAAATTCTTCAAGGTAAT	1104	
1105	GGGCTGAATACTTGTATAGTTTTAAGACTTAACAGGCCTTAAAAGGCCCCATGTTATCATAAAACGTCAT 1173	25,	n
1174	HindIII   1174 TGTTTAGAGTGCACCAAGCTTATAAAATGTAGCCAGGCCTTAAAAGACTTAACAGGCCTTAAAAGACTT :	742 1545	-1110
1243	1243 AACATTCCTTAAAAGGCCCCATGTTATCATAAACGTCATCGTTTTGAGTGCACCAAGCTAAATGTAGCC 1311	1311.	
1312	1312 AGGCCTTAAAAGACTTAACAGGCCTTAAAAGGCCCATGTTATCATAAAACGCCGTCGTTTTGAGTGCAC 1380	1380	

FIGURE 9 Page 2 of 6

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FIGURE 9 Page 3 of 6

2002 GTCTATTTGGTTTATTAGGCCAAACCAGAGGCGTTGAGAAGTGTCTAAGATAGTTAAGAAGCAGCTA 2070

~	1/7
2415	2484
2347 GAAGTAATTTTAGTATTAAGAGCAGCCAAGGCTTTGTTGGGTTTGTTGTTTTCATAATCTTCCTGTCAT 2415	2416 TITCTTTTTCTTTAAIGTGTCAAGCGACTCTGTTGGTTTAAAGTAGTATCTGTTTGCCATGGATCTCTC 2484

2			
HindII	×	GACTGAAAACTTTTGGTTTACACATGAAAGCTTGTTCTTGTTCTTTCT	2523
Sali	_	2485 ICTATITGTCGACTGAAAACTITTGGTTTA	2493

2623 ATTTATAAACAATCCTATTCACATTGTATACAGGTTATGATTATTCCCAATCAGCGTCAAAGAATCC 2691

FIGURE 9 Page 4 of 6

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FIGURE 9 Page 5 of 6 3589 ICTATCGTAGATGCTGTGACAAAAAAATTGTTTTATCGAAGATGAGAAACATGAGGCCTGTTCATGC 3657

BamHI

BamHI

3796 GGCTGTGATGTCGAACCAAACAGGCGACGATGAAGACAGTGATGATGACGTTTCCTTTGACTACAACGC 3864

BglII

3865 TGTCGGAAGCATTGGTCTCGCTGCCGGAAGATCT 3898

Page 6 of FIGURE

			•	-,, •	
•		69	138	1 207	276
		SduI AvaI AvaI    HgiJII HindIII         CTCGAGGCAGTCACTAACATGAAGTTTGACGAGGAGCCCAACTATGGGAAGCTTATTTCTCTTTTCGAT 39 50 36	XbaI     70 ACTCTAATTGAGCCGTGCGCTCTATATAGATTGATGGAGCTCTAAAGGTTGCTGGCTG	Ndel         TTTCTTGTTCATATACTTCTATAATATTCTCTGAAAGTGCTTCTTTTGGCATA 206	Ksp6321   TGTAGGTTGGGCAAAAACGAGGATTGCTTCTCAATTTGGAAGAGGATGAACAGCCGAAGAAAAA 245
	4325	SduI NIaIV HgiJII     ACGAGGAGCCCAACTATG 39	i gaccaattagaattgatg	SspI   CTTGTGTATAATATTCT 180	Ksp632I    SCTTCTCAATTTGGAAGA
	LENGTH =	GTCACTAACATGAAGTTTG	xbai                   	NdeI    Satargattaacttctaaa   150	GGCAAAACGAGGAAGATT
Lambda CGN1-2	NCG-186 Linear	XhoI AvaI I CTCGAGGCA(	70 ACTCTAATT	139 TTTCTTGTTC	.208 IGTAGGTIGG

FIGURE 10 Page 1 of 13

345	414	31/42	552
XhoII     305	MmeI EcoRV   1   346 AAAACATTTTTGCATATACACTTTGAAAGTTCCTCACTAACTGTGTAATCTTTTGGTAGATATCACTA 4	SduI MstI BclI HgiAI       415 CAATGTCGGAGAGAAA3GGCTGMNCANCATATACAAAAGGGAAATGAAGATGGCCTTTTGATTAGCTG 4	SduI HgiJII 1 484 TGTAGCATCAGCAGCTAATCTCTGGGCTCTTCATGGATGCTGGAACTGGATTCACTTCTCAAGTTTA 5

FIGURE 10 Page 2 of 13

621	069	759	828	897	996	1035
Cfr101 BbvII   1   553 TGAGTTGTCACCGGTCTTCCTACACAAGGTAATAATCAGTTGAAGCAATTAAGAATCAATTTGATTTGT 560 563	622 AGTAAACTAAGAAGTTACCTTATGTTTTCCCCGCAGGACTGGATTATGGAACAATGGGAAAAAAAA	Saci     TACTATATAAGCTCCATAGCTGGTTCAGATAACGGGAGCTCTTTAGTTGTTATGTCAAAAGGTTAGTGT   731	BbvII   760 ttagtgaataataaacttataccacaaagtcttcattgacttattatatata	829 GAACTACTTATTCTCAGCAGTCATACAAAGTGAGTGACTCATTTCCGTTCAAGTGGATAAATAA	898 GGAAAGAATTTTCATGTAACCTCCATGACAACTGCTGGTAATCGTTGGGGTGTGGTAATGTCGAGGA	Bell   967 ACTCTGGCTTCTCTGATCAGGTAGGTTTTTGTCTCTTATTGTCTGGTGTTTTTTTT
	. 🔾	•	,-	w	w	

FIGURE 10 Page 3 of 13

			•	33/4	v v		
1104	1173	Scal     1242   1242	1:311	1380	1449	1518	SspI     1587   1587
1036 CTAATATGATAAACTCTGCGTTGTGAAAGGTGGTGGAGCTTGACTTTTTTGTACCCAAGCGATGGGATAC	1105 ATAGGAGGTGGGAGAATGGGTATAGAATAACATCAATGGCAGCAACTGCGGATCAAGCAGCTTTCATAT	Tth11111   Scal	Xholl   	1312 TAGGACCTGAGAGCTTTTTGGTTTTTTTTTTCAGGACAAATGGGCGAAGAATCTGTACATTGCATCA	Aflii   1381 ATATGCTATGGCAGGACAGTGTGCTGATACACTTAAGCATCATGTGGAAAGCCAAAGACAATTGGAG 1449 1415	1450 CGAGACTCAGGGTCGTCATAATACCAATCAAAGACGTAAAACCAGACGCAACCTCTTTGGTTGAATGTA	SSPI    1519 ATGAAAGGGATGTGTTTGGTATGTACGAATAACAAAGAGAGATGGAATTAGTAGTAGAAATA 1587  1587
					• •	• •	• •

FIGURE 10 Page 4 of 13 ECORV

1588 TTTGGGAGCTTTTAAGCCCTTCAAGTGTGCTTTTTATCTTATTGATATCATCCATTTGCGTTGTTAA 1656

SspI

ECORI

Eco57I

1864 ICATTAAGTITITATTITCIGAAGTITTAAGTITTTACCTICTGTTTTGAAATATATCGTTCATAAGATG 1932

Page 5 of 13 FIGURE 10

2071 ATCTCCATTCTCACCTATAAATTAGAGCCTCGGCTTCACTCTTTACTCAAAACCAAAACTCATCACTACA 2139 METAlaAsnLysLeuPheLeuValSerAlaThrLeuAlaLeuPhePheLeuThr PmaCI SspI AflIII 2048 2053 SphI NspI [Avall1] SphI NspI 1971 2037 NdeI 2036 Ksp6321 2099 SecI Tth11111

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FIGURE 10 Page 6 of 13

	٠.	36/42	
2277	2346	2415	I Ksp632   C 2484 s s 1 1
Sall HindlI AccI AccI AccI	Tthillii Hindili Hindili NlaIV	Tth11111  AAGCAGGCAATGCAGTCCGGTAGTGGTCCAAGCTGGACCCTCGATGGTGAGTTTTGAAGACGAC LysGlnAlaMETGlnSerGlySerGlyProSerTrpThrLeuAspGlyGluPheAspPheGluAspAsp 2363	NlaIV Apal Gsul Hael NspBII Ks
22(	227	2347	2416

FIGURE 10 Page 8 of 13

2898	2967	3036	3105
	Spei Scai     CAGTACTAGTCTATTGAA 2 2954 2955	ATGATAAAAGATCATCCA 3	ATTTTTGTATTCATTAAA 3
GAAGTTTTTTTTACTCTTGCTAC1	Vsp.i         AACAATTAATTCACAATGAAG   2929	IGTCTAATTAAGCCTTCAAGGCTAGTG	Xholl NlalV NalV Bcll Bcll       ATGGGATCCAACAAAGACTCAAAATCTTAAAAAAAAAAA
Acci   2830 TACTCCGTAGACGGTAATAAAGAGAAGTTTTTTTTTTTACTCTTGCTACTTTCCTATAAAGTGATGAT 2838	Spei Vspi         2899 TAACAACAATACAATTAATCTATATTCACAATGAAGCAGTACTATTGAA 2967 2954	NSPI Afliii     2968 CATGTCAGATTTTCTTAATGTCTAATTAAGCCTTCAAGGCTAGTGATAAAAGATCATCCA 3036 2968 2972	Xholl NlalV BamHI   1 3037 ATGGGATCCAACAAGACTCAAATG 3043

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FIGURE 10 Page 9 of 13

3106 TTATGCAAGTGTTCTTTTATTTGGTGAAGACTCTTTAGAAGCAAAGAACGACAAGCAGTAATAAAAAA 3174 3139 Tth111 BbvII

3175 ACAAAGTTCAGTTTTAAGATTTGTTATTGACTTATTGTCATTTGAAAATATAGTATGATATTAATATA 3243

VspI Tth1111I

3250

Tth1111I

NdeI

3382 CTGAAGAAAAGATAAGTGAGCTTCGAGTTTCTGAAGGGTACGTGATCTTCATTTCTTGGCTAAAAGCGA 3450 Eco57I 3434 Eco57I 3404

3451 ATATGACATCACCTAGAGAAAGCCGATAATAGTAAACTCTGTTCTTGGTTTTTGGTTTAATCAAACCGA 3519

Page 10 of 13 FIGURE 10

FIGURE 10 Page 11 of 13

	•	• •		
3588	3657	3726	3795	3864
NdeI NdeI    	Cfr10I   3589 GTTGTAAACCGGTATTTCATTTGGTGAAACCCTAGAAGCCAGCC	NlaIV HindII HgicI BspHI               1     3717   3718	Eco31I PmaCI Ksp6321 	3796 CGGCGG5MNTTTGGTGGCGGCGGCGGACGTTTTGGTGGCGGCGGTGGACGTTTTGGTGGCGGCGGTGGA 3864
Cfr101   3520 ACCGGTAGCTGAG 3521	Cfr101   589 GTTGTAAACCGGTA	3658 AACGAGAAGTCAC	3727 CAAATAAAACCCG	796 CGGCGGSMNTTGC
(7)	m	m	<b>6</b>	3

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ECORV

HindIII

Aflii

3934 ICGAAICTIATICTIGCTCTGCTCGTTGTTTTACCGAIAAAGCTTAAGACTTTATTGATAAAGTTCTCA 4002

3974

4003 GCTTTGAATGTGAATGAACTGTTTCCTGCTTATTAGTGTTCCTTTGTTTTGAGTTGAATCACTGTCTTA 4071

GCACTTTTGTTAGATTCATCTTTGTGTTTAAGTTAAAAGGTAGAAACTTTGTGACTTGTCTCCGTTATG 4140

HpaI HindII

Tth1111I

XhoII

4210 GACCAAGCTCTCTCAGGCGAAGATCCCTTACTTCAATGCCCCAATCTACTTGGAAAACAAGACACAGAT 4278

FIGURE 10 Page 12 of 13

42/42

FIGURE 10 Page 13 of 13

Sall PstI

## INTERNATIONAL SEARCH REPORT

International Application "a. PCT/US91/01746 I. CLASSIFICATION OF BJECT MATTER (if several classification symbols apply, indicate all) 6 According to International Patent Classification (IPC) as to the help of 12 figure and IPC IPC(5): C12N 1/21, 15/29, 15/82; CO7H 15/12 figure and IPC U.S. CL.: 435/172.3, 240,4, 252.3; 536/27 II. FIELDS SEARCHED Minimum Documentation Searched 7 Classification System Classification Symbols 435/172.3, 240.4, 252.3 U.S. 536/27 800/205, DIG.69 Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched USPTO AUTOMATED PATENT SYSTEM: DIALOG FILES BIOTECH AND PATENTS. SEE ATTACHMENT FOR SEARCH TERMS III. DOCUMENTS CONSIDERED TO BE RELEVANT Citation of Document, 11 with indication, where appropriate, of the relevant passages 12 Relevant to Claim No. 13 SEE ATTACHED PAGES Special categories of cited documents: 10 "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle of theory underlying the invention "A" document defining the general state of the art which is not considered to be of particular relevance. earlier document but published on or after the international filing date "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "L" document which may throw doubts on priority claim(s) or which is-cited to establish the publication date of another citation or other special reason (as specified) document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "O" document referring to an oral disclosure, use, exhibition or other means document published prior to the international filing date-but later than the priority date claimed "A" document member of the same patent family IV. CERTIFICATION Date of the Actual Completion of the International Search Date of Mailing of this International Search Report 07 AUG 1991 24 June 1991 Signature of Authorized Officer International Searching Authority RO/US

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1	acyl-acyl carrier protein thioesterase from		
ŀ	maturing seeds of safflower*: pages 12141-12147		
1	See entire document.		
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ategory .	MENTS CONSIDED TO BE RELEVANT (CONTINUED FROM THE SECO) SHEE Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
		Notati to Claim No
х	Proceedings of the Flax Institute USA; Volume 41, Number 3; Downey et al; "Genetic control of fatty acid composition in oilseed crops"; pages 1-3. See entire document.	23-33, 38-45
X Y	EP. A,0323753 (WONG et al) 12 July 1989. See entire document.	23-29, 31-33 38-39, 41-45 30, 40
X Y	Journal of the American Oil Chemists Society; Volume 61, Number 1; Issued January 1984; Wilcox et al; "Genetic alteration of soybean oil composition by a chemical mutagen"; pages 97-100. See entire document.	23, 25-27, 29, 31, 33, 38-45 30, 40
<u>X</u>	Journal of the American Oil Chemists Society; Volume 59, Number 5; Issued May 1982; Wolf et al: "Effect of temperature on soybean seed constituents: oil, protein, moisture, fatty acids, amino acids and sugars"; pages 220-222. See entire document.	23, 25-27, 29, 31, 33, 38-45 30, 40
Υ .	Lipids; Volume 4, Number 6; Issued 1969; Inkpen et al; *Desaturation of palmitate and stearate by cell-free fractions from soybean cotyledons*; pages 539-543. See entire document.	30, 40
Y	The Journal of Biological Chemistry; Volume 241; Issued 1966; Nagai et al; "Enzymatic desaturation of stearyl acyl carrier protein"; pages 1925-1927. See entire document.	30. 40
<b>X</b> .	The Journal of Heredity; Volume 80, Number 3; Issued March 1989; Moore et al; "The inheritance of high cleic acid in peanut"; pages 252-253. See entire document.	23, 25-27, 29 31, 33, 38-45 30, 40
<u>x</u>	Crop Science; Volume 24; Issued November- December 1984; Carver et al; "Developmental changes in acyl-composition of soybean seed selected for high oleic acid concentration"; pages 1016-1019. See entire document.	23.25-27,29 31.33.38-45 30,40
XY	Bodman et al., "Soybeans and Soybean Products: Processing of edible soybean oil" published 1951 by Interscience Publishers, Inc. (N.Y.), pages 649-725, see only pages 702-709.	31 and 33 32